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Drug Repurposing

Molecular Aspects and Therapeutic Applications

Trends in Molecular Aspects and Therapeutic Applications of Drug Repurposing for Infectious Diseases

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Abstract

The pharmaceutical industry has undergone a severe economic crunch in antibiotic discovery research due to evolving bacterial resistance along with enormous time and money that gets consumed in *de novo* drug design and discovery strategies. Nevertheless, drug repurposing has evolved as an economically safer and excellent alternative strategy to identify approved drugs for new therapeutic indications. Virtual high throughput screening (vHTS) and phenotype-based high throughput screening (HTS) of approved molecules play a crucial role in identifying, developing, and repurposing old drug molecules into anti-infective agents either alone or in synergistic combination with antibiotic therapy. This Chapter briefly explains the process of drug repurposing/repositioning in comparison to *de novo* methods utilizing vHTS and HTS technologies along with 'omics- and poly-pharmacology-based drug repurposing strategies in the identification and development of anti-microbial agents. This Chapter also gives an insightful survey of the intellectual property landscape on drug repurposing. Further, the challenges and applications of drug repurposing strategies in the discovery of anti-infective drugs are exemplified. The future perspectives of drug repurposing in the context of anti-infective agents are also discussed.

Keywords: drug repurposing, repositioning, poly-pharmacology, anti-infectives, HTS

1. Introduction

Antibiotic resistance is a major threat that may lead to approximately 10 million deaths per year by 2050 [1]. Nevertheless, pharmaceutical companies' entire economic model for antibiotic drug discovery has clashed with the low profitability index. An estimated cost of developing an antibiotic in 2017 was nearly US $1.5 billion, whereas the average revenue generated per year is nearly US $46 million, which cannot be justified in any way [2]. Therefore, in an attempt to accelerate the identification of potential and safe anti-infective drugs, reduce discovery research expenses,

and minimize drug development timeline, "drug repurposing" and/or "drug repositioning" has arisen as an excellent alternative approach because the developer already has the complete pharmacological and toxicological data of the drug candidate from preclinical and clinical trials. Drug repurposing and/or repositioning simply mean new treatment indication or pharmacological use of an old drug [3]. For example, Aspirin, the first-ever drug repurposed, was originally indicated as an analgesic but later repurposed for various pharmacological effects such as anti-platelet in cardiovascular events [4].

"Drug repurposing," "drug repositioning," and "drug rescuing" are the terms generally used interchangeably; however, these terms may slightly differ from each other. Drug repurposing means, "approved drug for one disease is identified potentially useful and repurposed in another disease" such as aspirin, whereas drug repositioning explains a situation when "an approved drug for one disease is used as a template and derivatized to a different form for use in another disease" [5, 6]. Nevertheless, drug rescuing is the term given to the concept where "the clinically failed or market abandoned drugs for one clinical indication is rescued or used for another clinical indication" such as thalidomide which was banned initially but later rescued to multiple myeloma [5]. However, the ultimate goal remains the same and that is "repurposing of old drugs for new diseases."

2. Need for drug repurposing

Nobel Laureate Sir James Whyte Black (1924–2010) had once said that "the most fruitful basis for the discovery of a new drug is to start with an old drug" [7]. However, the systematic screening approach introduced by Paul Ehrlich became the cornerstone of antibiotic search strategies for pharmaceutical industries and along with further advancement in *de novo* drug design methods, various potential novel classes of antibiotics were discovered. Nevertheless, the rate of discovery of a novel

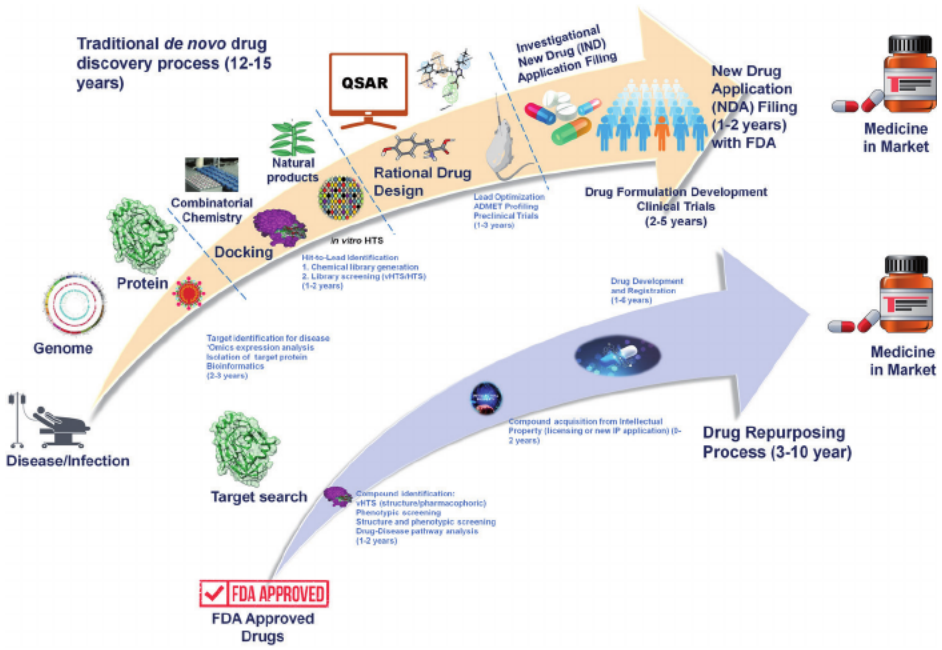

Figure 1.
Traditional de novo *drug discovery process versus drug repurposing process.*

class of drugs suddenly dropped by 1970 with the increasing rate of resistance [8]. Even with all the scientific tools of traditional methods of drug discovery such as 'omics (genomics, proteomics, and metabolomics), virtual high throughput screening (vHTS), phenotypic, and whole cell-based high throughput screening (HTS), no new class of antibiotics are getting discovered [8]. On the contrary, there is an overall increase in the expenses leading to a collapse in the economic model of antibiotic drug discovery research [2]. Therefore, a change in the financial models is required to translate scientific advances into clinically approved antibiotics [9]. Drug repurposing is the best possible way to escape from this dilemma and reposition the drug candidates from the approved pharmacopeia. Drug repurposing offers great advantages over traditional drug discovery methods such as no chemical optimization and reduced developmental risk because the drug candidates have often been through several stages of preclinical and clinical trials and therefore have well-known toxicological safety and pharmacokinetics profile. Even formulation stages and bulk manufacturing are also bypassed, enabling a shorter route to the market [3]. A comparison of traditional *de novo* drug discovery versus drug repurposing is summarized in **Figure 1**.

3. Intellectual property landscape in drug repurposing

The drugs may either be on-market (ONM) or off-market (OFM) drugs. Further, the ONM drugs may be on-patent (ONP) or off-patent (OFP) drugs. As per the latest version of the United States Food and Drug Administration-Orange Book (US FDA-OB), 1577 drugs are ONM drugs and 1543 drugs are OFM drugs. Out of 1577 ONM drugs, 1142 drugs lack patent/exclusivity claims and could be utilized for drug repositioning projects [10]. Nevertheless, obtaining patent protection for known drugs can be a challenging task. The repurposed drug can be patented in the United States if the drug constitutes patentable subject matter under 35 U.S.C. § 101. According to 35 U.S.C. §, 101 repurposed drugs may be patented provided its new indication or use has not been published before. Nevertheless, the eligibility of patentability of "therapeutic use" varies between jurisdictions from country to country [11]. While the patent based on "therapeutic use" is possible in the United States and some other countries, it is not permitted in India. Therefore, another approach to obtain a patent for previously known drugs in India is to draft claims for novel pharmaceutical formulations. On the contrary, if a drug to be repurposed is still under patent protection, then that drug can either be acquired or in-licensed from the patentee. Hence, patent protection of repurposed drugs for new indications is possible. However, initial experimentation should establish the usefulness of the drugs along with robust invention disclosures and detailed formulation applications may be directed to the patent office [12].

4. Strategies involved in drug repurposing

Drug repurposing in infectious diseases involves different strategies by integrating both vHTS and HTS methodologies to identify a drug molecule, a microbial target, and an immunopathological pathway to fight against an infectious pathogen. The various strategies involved are (i) computer-aided (structure-based [13] and ligand-based pharmacophoric [14]) repurposing, (ii) phenotype-based HTS aided repurposing [15], (iii) 'omics-based drug repurposing [16], (iv) drug-disease biological pathway analysis [17, 18], (v) poly-pharmacology-based drug repurposing [19, 20], and (vi) serendipity [21], which are summarized in **Figure 2**.

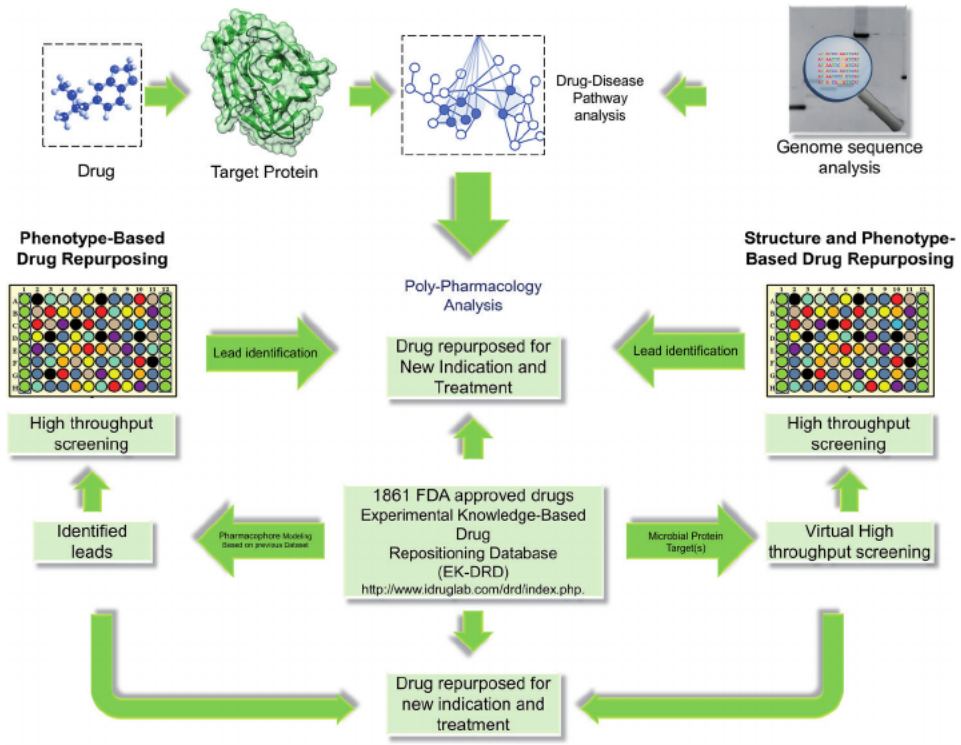

Figure 2.
Strategies involved in drug repurposing.

However, the plausible drugs for anti-microbial repurposing may fall into three different evaluating scenarios and two different approaches, namely, "on-target repurposing" and "off-target repurposing" [22] as shown in **Figure 3**.

4.1 Computer-aided drug repurposing

vHTS is an efficient approach to identify compounds for drug repurposing. Where vHTS is a generalized term for different screening filters, it is categorized under two broad classes of virtual screening for drug repurposing, that is, (i) structure-based drug repurposing and (ii) ligand-based pharmacophoric repurposing.

4.1.1 Structure-based drug repurposing

Protein data bank (PDB) is the largest compilation of structural data on microbial target proteins. Presently, there are 62,402 structural deposits related to bacterial target proteins and 9653 structural deposits related to viral target proteins in PDB.

Further, nearly 60% of these proteins are complexed with a biologically relevant ligand, which provides information about the shared binding sites and amino acids of the target site involved in intramolecular interactions with the ligand. These proteins are utilized for structure-based drug repurposing by virtual screening (docking studies) the drugs for repurposing in comparison to the ligand. The ligand in comparison could either be the one that is already complexed at the target site or any other approved drugs available as a particular modulator of the target site. The selection of screened drugs for repurposing is completely based on scoring and drug interactions. To complement the structures available in the PDB, another method used for structure-based screening is called homology modeling. Homology

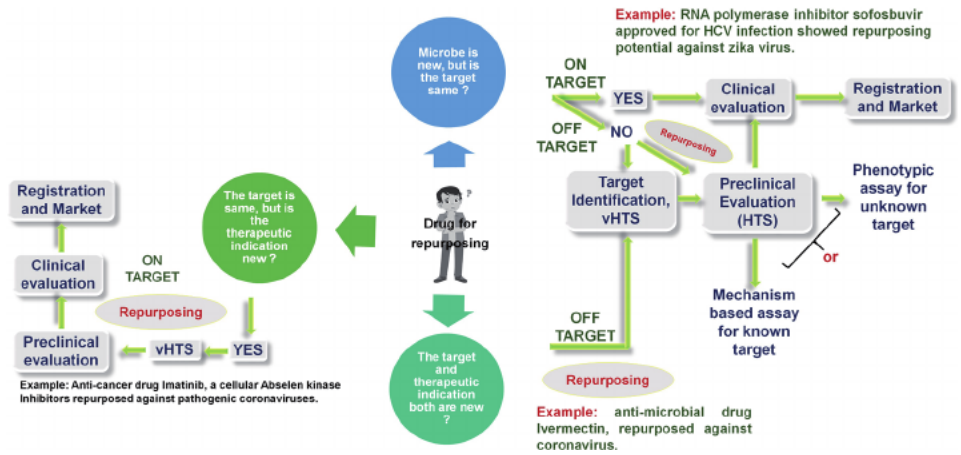

Figure 3.
Various scenarios and approaches in drug repurposing.

modeling can generate 3D structures of even those proteins whose structures are difficult to obtain through X-ray crystallography. Apart from these sources, there are other databases of high-quality 3D protein models, such as SWISS-MODEL Repository (SMR) to support structure-based drug repositioning pipelines [13].

4.1.2 Ligand-based drug repurposing

In the absence of structural information about the microbial target protein from source, structural databases, or homology modeling, the structure-based repositioning and discovery efforts are hampered. Nevertheless, there are other virtual screening methods such as ligand-based screening methods, which can be employed for drug repurposing. The process involves the generation of a ligand-based mathematical "QSAR (quantitative structure–activity relationship) model" and ligand-based 3-dimensional (3D) "pharmacophore fingerprint" using in-house or approved microbial target site inhibitors as the active set I. The drugs sought to be repurposed are arranged in set II and screened using the derivatized models for their optimum physicochemical descriptors and/or conformational search for active pharmacophore. The potential molecules through ligand-based screening approach will be shortlisted for phenotype-based HTS studies [14].

4.2 Phenotype-based HTS-aided repurposing

There are various unexplored targets and pathways within the complexity of the microbial intracellular mechanisms along with the identified targets. The drugs for repurposing may be screened for known off-target, on-target, and unknown targets using HTS (**Figure 3**). Mechanism-based biochemical assays may be carried out for known off-target and on-target screening of drugs employing specific proteins such as enzymes in the assay. However, the unknown off-target screening can be carried out through phenotype cell-based HTS assays, so that the multiple targets can be screened to conclude the efficacy of repurposed drug related to its pharmacodynamic status, heterogeneity, biomarker readout, membrane permeability, and cytotoxicity. Further, the phenotype-based assays may be carried out using two-dimension (2D) and three-dimension (3D) approaches. The 2D approach is a traditional cell-based HTS that is carried out on cultured cells propagated in 2D on plastic surfaces

optimized for cell culture. Anti-infective screening for drug repurposing traditionally utilizes a 2D cell-based HTS approach. However, this approach is not suitable for accessing the drug resistance status in antimicrobials. Thus, for drug repurposing or discovery, bioengineered 3D cell culture technology that closely resembles the in vivo cell environment is now being pursued [15].

4.3 'Omics-based drug repurposing

Omics technology comprises various approaches such as genomics, transcriptomic, proteomics, and metabolomics. The genomics and transcriptomic approaches analyze the gene pattern and mRNA sequence of a pathogen before and after exposure to a drug under consideration for repurposing. The study of the gene expression at the transcription level helps researchers to predict possible metabolic pathways of microorganisms, genomic mutation leading to drug resistance, and potential targets. Further, large-scale microbial gene expression studies may be carried out using advanced microchip technology. The proteomic approach evaluates the overall protein expression profile of the entire organism pre- and post-exposure to an antimicrobial agent under various environmental conditions. It helps identify drugs that may be repurposed for plausible new targets with the least chances of resistance and novel mechanism of action. In contrast, metabolomics involves the analyses of metabolites, and biological/molecular substrates present in a pathogen at a particular time interval. Further, exometabolomics, also known as "metabolic footprint" measures charged or polar molecules being consumed or released by an organism as a secondary metabolite. Sound knowledge of metabolomics can predict the alternative mechanism or pathway during drug resistance, and synergy in combination therapy. Hence, 'omics technologies have transformed the anti-infective drug discovery by generating an unparallel amount of data on potential antimicrobial targets and their resistance from the array of biological libraries. The unique signature (characteristics) of a disease and its co-relationship with a drug can be derivatized using 'omics technologies and drug databases, respectively, such as CARD (Comprehensive Antibiotic Research Database), ARDB (Antibiotic Resistance Genes Database), and NDARO (National Database of Antibiotic-Resistant Organisms) [16].

4.4 Drug-disease biological pathway analysis

Traditionally, computer-aided approaches were mainly aimed toward target and drug molecules involving structure-based drug design. However, it has also been employed toward the assessment of biological pathways, and mechanisms of drugs through network systems to formulate the correlation between drugs and disease pathways for possible drug repositioning. The scientific data over the drug-disease pathways network may be designed using various databases such as NCBI, MMDB, GEO, and PubChem. Using this approach, Yang et al. generated three network-based systems between cardiovascular diseases, diabetes mellitus, and neoplasms to establish the drug-disease biological pathway correlation and to predict possible drugs for repositioning. Similarly, Pan et al. studied 16 FDA-approved drugs for possible drug repurposing by using a drug-disease pathway-based approach. Their approach involved the analysis of the drug, protein, and corresponding gene target with affected gene expression level after drug treatment [17, 18].

4.5 Poly-pharmacology-based drug repurposing

The "single drug, single target" approach is an oversimplified disease mechanism which is in fact, a complex sub-network of the underlying distorted

physiological pathway within the interactome. In contrast, network pharmacology considers disease a casual mechanism within the "diseasome cluster" and treats by identifying the synergistic co-targets leading to reduced dose and side effects of the drug. Similarly, "polypharmacology" is the concept of designing or utilizing pharmaceutical agents that can synergistically act on multiple targets or disease pathways. Thus, the drugs which are poly targeting allow a broader impact not only in the early stages of drug discovery but in drug repositioning as well. Various polypharmacology- and network pharmacology-based databases have been published which are employed to develop polypharmacology-based drug repurposing predictions. Polypharmacology apart from the concept also incorporates the use of computational fingerprinting such as structure-based polypharmacology and ligand-based polypharmacology similar to SBDD and LBDD [19, 20].

4.6 Serendipity

"Serendipity," a term used by medical writers for almost 50 years, was originally coined in 1754 by Horace Walpole in an allusion to an ancient oriental legend of the "Three Princes of Serendip." Today serendipity means, "discoveries not purposely searched for" [21]. However, this term has become one of the methods for discoveries. A thorough survey (via social media platforms) based on medical questions and answers can form a database for the serendipity approach in drug repurposing. This approach can be best understood by various examples where patients taking medication "A" for a specific ailment but suffering from comorbidities have claimed to have found relief from the comorbid disease as well. For example, a patient taking hydrochlorothiazide prescribed for hypertension found relief in kidney stones. However, there is a logical scientific connection between the two conditions. Hydrochlorothiazide is an antihypertensive drug that functions through its diuretic properties (increased urine production and flow) leading to either dissolution or removal of small kidney stones. Similarly, a second example is of a 41-year-old woman with depression and psoriasis and was under treatment for depression with sertraline. She noticed that with sertraline her psoriatic lesions started disappearing. However, scientifically these two conditions are also correlated as psoriasis being an autoimmune disorder having a direct impact on psychosocial factors leading to depression and periodical inflammatory lesions. The main limitations of this method can be questioned in terms of its credibility as these databases are just an output of a questionnaire where other factors such as lifestyle change and environmental factors too might have played an important role. However, the conclusions may be evaluated using drug-disease pathway analysis and other drug repurposing strategies [22].

5. Challenges in drug repurposing

Traditional drug discovery is a time-consuming (10–17 years) process that bears failure risk and huge investment. In this regard, drug repurposing strategy has a lower rate of failure and is found to be safe in early preclinical and clinical trials, thus reducing the cost and time spent during formulation development, safety, and efficacy studies. However, the major challenges in drug repurposing could be (i) untoward side effects due to higher dose of the nonantibiotic drug repurposed for infectious diseases to show the required therapeutic effect and (ii) variation in the pharmacokinetic profile of the drug after off-target repurposing.

6. Therapeutic applications of drug repurposing in infectious diseases

Drug repurposing strategy recently identified that the anthelmintic drug niclosamide (NCL) is a strong inhibitor of the 3OC12 – HSL-dependent QS system in *Pseudomonas aeruginosa* by inhibiting the LasR-dependent signaling leading to reduced virulence, and attenuated *P. aeruginosa*. Pulmonary administration is an ideal route to treat respiratory infections but the major obstacle in pulmonary administration of NCL was the achievement of appropriate particle size and its poor dissolution properties in alveolar fluids due to hydrophobicity. Hence, therapeutic applications of nanotechnology were employed to formulate NCL nano dry powders using high-pressure homogenization and spray drying technologies. Thus, repurposed drugs based on their pharmacokinetic profile may be modified in the form of nano-suspensions enhancing the drug's potential for the treatment of infectious diseases [23]. Similarly, synergistic drug combination along with antibiotics is a useful therapeutic option for various repurposed nonantibiotic drugs showing less potential against infections leading to reduced chances of attaining antibiotic resistance [24, 25].

7. Repurposed drugs for infectious diseases

Few examples of directed repurposed drugs for bacterial, viral, and fungal diseases are summarized in **Table 1**. Drugs such as Auranofin, Celecoxib, Clomiphene, and Finasteride have been repurposed for several bacterial infections. Similarly, Remdesivir, Favipiravir, Lopinavir-Ritonavir, Ivermectin, Ribavirin, Interferon, and Hydroxychloroquine have been repurposed for COVID-19. Haloperidol, Aripiprazole, Alexidine dihydrochloride, Pentamidine, bifonazole, and Sulfonamide drugs have been repurposed for fungal infections.

8. Conclusion

The growing number of resistant infectious agents is a threat to the world. Various screening strategies such as vHTS, HTS (phenotypic cell-based 2D/3D screening), and therapeutic approaches (nanotechnology, synergistic combinations) for drug repurposing may be employed for rapid identification and formulation of new therapeutics against infections. These approaches are especially useful during emerging outbreaks and pandemics of infectious diseases such as MERS, SARS, SARS-CoV-2, and Ebola viruses because it is highly impractical to develop vaccines and therapeutic agents in a short period. Nevertheless, there is an important question that needs to be addressed by the scientist working toward a drug repurposing approach. What if the existing pharmacopoeia for repurposing will get exhausted one day?

9. Future perspectives

The boom in drug repurposing strategies may occupy the existing drugs from pharmacopoeia and the drug bank may get exhausted for further repurposing. Therefore, pharmaceutical companies with advanced biological and technological expertise should invest in biodiversity-oriented drug discovery programs to discover and develop early-stage new pharmacophoric compounds

Sr. No.	Drug repurposed	Clinical indication	Target pathogen and mechanism of action
Repurposed drugs for bacterial infections			
1.	Auranofin	Rheumatoid arthritis	*Staphylococcus aureus*: inhibition of DNA/protein synthesis, and downregulation of toxin production.
2.	Celecoxib	Inflammation	*S. aureus*, *Bacillus anthracis*, *B. subtilis*, and *Mycobacterium smegmatis*: inhibition of bacterial DNA, RNA, protein synthesis, and cell wall.
3.	Clomiphene	Fertility	*S. aureus*: inhibition of undecaprenyldiphosphate synthase involved in the synthesis of a teichoic acid wall.
4.	Finasteride	Prostate hyperplasia	*Candida albicans*: inhibition of filamentation.
5.	Clotrimazole and Miconazole	Fungal infection	*P. aeruginosa*: inhibition of the pqs activity through the possible inactivation of 2-alkyl-4-quinolones (AQ) production or reception.
Repurposed drugs for viral infections			
6.	Ivermectin	Anthelmintic	SARS-CoV-2: acts blocking the nuclear transport of viral proteins
7.	Nitazoxanide	Parasitic and viral infection	Influenza virus: inhibition of the pyruvate: ferredoxin/flavodoxinoxidoreductase cycle.
Repurposed drugs for fungal infections			
8.	Haloperidol	Antipsychotic agent	*C. albicans*: inhibition of filamentation, melanin production, and biofilm formation.
9.	Aripiprazole	Antipsychotic agent	Inhibition of biofilm formation and hyphal filamentation.

Table 1.
Directed repurposed drugs for infections [26–28].

and fill their anti-infective pipelines while still taking the advantage of drug repurposing. Further, the advancement in nanotechnology may lead us to design better therapeutic formulations of repurposed drugs targeting pulmonary infections such as multidrug-resistant tuberculosis. Drug repurposing raises several concerns in terms of quality and ethical integrity of preclinical and clinical research specially during emergency pandemic situations such as COVID-19 involving accelerated drug approval based on statistical exploration of small, scientific data with the real-world population. This issue may not only increase the chances of adverse events; also, if the drug is withdrawn, the pharmaceutical industry may lose public confidence over healthcare needs. According to patent regulations, there are no safeguards for Intellectual property (IP) protection of drug development through the repositioning method. IP protection for repositioned drugs is limited. If the current evidence is not sufficient and does not meet the standards of according to regulatory guidelines, regulatory agencies such as the FDA or EMA, further preclinical and/or clinical studies may be necessary.

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Evaluation of Drug Repositioning by Molecular Docking of Pharmaceutical Resources to Identification of Potential *SARS-CoV-2* Viral Inhibitors

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Abstract

Unfortunately, to date, there is no approved specific antiviral drug treatment against COVID-19. Due to the costly and time-consuming nature of the de novo drug discovery and development process, in recent days, the computational drug repositioning method has been highly regarded for accelerating the drug-discovery process. The selection of drug target molecule(s), preparation of an approved therapeutics agent library, and in silico evaluation of their affinity to the subjected target(s) are the main steps of a molecular docking-based drug repositioning process, which is the most common computational drug re-tasking process. In this Chapter, after a review on origin, pathophysiology, molecular biology, and drug development strategies against COVID-19, recent advances, challenges as well as the future perspective of molecular docking-based drug repositioning for COVID-19 are discussed. Furthermore, as a case study, the molecular docking-based drug repurposing process was planned to screen the 3CLpro inhibitor(s) among the nine Food and Drug Administration (FDA)-approved antiviral protease inhibitors. The results demonstrated that Fosamprenavir had the highest binding affinity to 3CLpro and can be considered for more in silico, in vitro, and in vivo evaluations as an effective repurposed anti-COVID-19 drug.

Keywords: bioinformatics, protein–peptide interactions, biological targets, drug development, 3CLpro inhibitor, biological computation, drug design

1. Introduction

The Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (*SARS-CoV-2*), signifies a pandemic threat to international health, with so far nearly 5 million deaths worldwide [1]. Notwithstanding mass vaccination worldwide by emergency approved vaccines such as Pfizer-BioNTech, Janssen, and Moderna, COVID-19 still poses a threat to human health. Furthermore, with the emergence of new mutant strains of SARA-CoV-2 as well as

a significant decrease in the vaccine's efficacies, introducing of new treatment strategies is urgently needed. Therefore, recently many international efforts have been planned for introducing suitable vaccines as well as effective therapeutics [2, 3].

Generally, time is a vital factor in the pandemic condition, so that, rapid detection, vaccination, and treatment methods can significantly reduce mortality. De novo drug discovery and development for lesser-known diseases such as COVID-19 is costly and tedious. Consequently, alternative methods such as the computational drug repurposing approach can accelerate the discovery of new drugs. In this regard, several pipelines have been introduced for in silico drug repositioning against COVID-19. Lately, molecular docking as a popular bioinformatics method has been highly regarded as the core of the most drug repositioning process to achieve effective drug candidates to combat COVID-19 [4–6]. In this chapter, we discussed new advancements and challenges in drug repositioning by molecular docking of pharmaceutical resources to the identification of potential *SARS-CoV-2* viral inhibitors.

2. Origin and pathophysiology aspects of COVID-19

SARS-CoV-2 was firstly discovered in the Huanan Seafood Wholesale market in Wuhan, China on 12 December 2019 [7]. Subsequent to the extensive outbreak of the virus infection, on March 11, 2020, the World Health Organization (WHO) announced the COVID-19 pandemic. As of 27 August 2021, the total number of cases of *SARS-CoV-2* confirmed globally by WHO are 214,468,601 with 4,470,969 reported deaths (https://covid19.who.int/). As per the reports of WHO, the mortality rate of COVID-19 is around 3.7% [8]. Although the host of *SARS-CoV-2* is still indistinct, it is assumed the virus has bats or pangolins origin. However, the main theory suggests that the virus was transmitted to humans from an intermediate host. The virus is mainly transmitted among the individuals through droplet infection, contact routes, and rarely through the feces of the infected patients and mother to child post-childbirth. Fever, cough, fatigue, diarrhea, headache, hemoptysis, dyspnea, acute respiratory distress syndrome, cardiac injury, and lymphopenia are known clinical manifestations of COVID-19. COVID-19 infection can be divided into three phases including the virus replication and appearance of mild signs, the emergence of respiratory symptoms and simulation of the adaptive immune system responses, and the third phase causing hyper-inflammation. Expression of the ACE2 (angiotensin-converting enzyme 2) protein (as the major receptor molecule for the virus) by renal tubular cells, liver cells and testicular cells may the kidney, liver, and testicular tissue damages also observed in the COVID-19 patients [1, 9, 10].

3. Molecular biology of *SARS-CoV-2*

SARS-CoV-2 belongs to beta coronaviruses and has a round or elliptic form, with an approximate diameter of 60–140 nm. The virus genome is an around 30 Kb positive-sense, single-stranded RNA, which encodes four structural proteins including S protein (Spike), E protein (Envelope), M protein (Membrane), and N protein (Nucleocapsid), and several accessory proteins or nonstructural proteins, namely, NSP1 to NSP16 [11]. S protein is 150 kDa, acts as an anchor on the virus envelope, and consists of three domains including the outer N-terminal domain having unit S1 and S2, a cytoplasmic C-terminal domain, and a transmembrane domain. M protein is 25–35 kDa, a transmembrane glycoprotein type III and the most abundant protein on the surface of the virus. Based on the bioinformatics analysis, the protein can play a role in the virus entry into the host cell and its RNA maturation.

Protein name	Length (amino acid)	Role	References
NSP1	180	Host translation inhibitor and also degrade host mRNAs	[1]
NSP2	638	Binds to prohibitin 1 and prohibitin 2	[2]
NSP3	1945	Responsible for release of NSP1, NSP2, and NSP3	[3]
NSP4	500	Viral replication-transcription	[4]
NSP5	306	Cleaves at multiple distinct sites to yield mature	[5]
NSP6	290	Induces formation of ER-derived autophagosomes	[6]
NSP7	83	Forms complex with NSP8 and NSP12 to yield the RNA polymerase activity of NSP8	[7]
NSP8	198	Makes heterodimer with NSP8	[8]
NSP9	198	bind to helicase	[5]
NSP10	139	Unknown	[5]
NSP11	13	Unknown	[5]
NSP12	932	Replication and methylation	[9]
NSP13	932	A helicase core domain	[10]
NSP14	527	Exoribonuclease activity a	[5]
NSP15	346	Mn(2 +)-dependent endoribonuclease activity	[5]
NSP16	298	Methyltransferase	[11]

The role(s) of NSP10 and NSP11 is(are) still not well understood.

Table 1.
Description of various roles of non-structural proteins from SARS-CoV-2.

N protein is a 43–50 kDa nucleocapsid structural protein and has a vital role in attaching and assembling the virus genome to the matrix of the ribonucleoprotein. The E protein is 8.4–109 kDa and is recognized as a small hydrophobic protein. The protein contributes to viroporin activity, virus assembling, and the virus budding process. Based on the results of several studies, the nonstructural proteins encoded by genes positioned within the 5′-region of the virus genome, have a wide range of roles from host translation inhibition by NS1 to viral replication-transcription NS4 [12, 13]. The main roles of the known nonstructural proteins of *SARS-CoV-2* are summarized in **Table 1**.

4. Antiviral molecular targets and drug development strategies against COVID-19

Generally, a probable antiviral drug target is a molecule (often a protein) with a vital role in the life cycle of the planned virus [14, 15]. Accordingly, to date, several structural and accessory proteins from *SARS-CoV-2* have been subjected to the drug-discovery process. Consistent with the approved information about the *SARS-CoV-2* life cycle, eight steps including virus binding, fusion to host cell, RNA release, translation, proteolysis, replication and translation, viral assembly, and release could be planned to investigate potential anti-COVID-19 drugs. Among the mentioned steps, virus attachment and entry, proteolysis, and replication have received more attention due to more available data about the key proteins in the steps as well as the high similarity of these steps between coronaviruses [16, 17].

In the following sections, the key steps in *SARS-CoV-2* life cycle are discussed in the light of drug development against COVID-19.

4.1 Virus attachment and entry

The trimeric *SARS-CoV-2* spike glycoprotein has a crucial role in the virus attachment and entry. The glycoprotein constituent monomer comprises two subunits, S1 and S2. The S1 encompasses the N-terminal domain (NTD) and the RBD, which is accountable for interacting with ACE2. Therefore, RDB is considered an effective drug target for discovering therapeutic agents such as neutralizing antibodies [18]. In **Table 2**, some anti-RBD antibodies are listed. The results of some studies demonstrated potent therapeutic and prophylactic abilities of anti-RDB antibodies in cell culture or animal model systems. In this regard, Gao et al. demonstrated that a potent COVID-19 antibody, BD-368-2 has significant prophylactic effectiveness in *SARS-CoV-2*-infected hACE2 mice at a dose of 20 mg/kg [24]. Similarly, another study confirmed both prophylactic and treatment activities of CB6 antibody in a dose of 50 mg/kg [25]. The ability of COV2-2130 to reduce the viral burden and levels of inflammation has also been approved [26]. Furthermore, besides the introduced antibodies, several small molecules such as salvianolic acid, arbidol, dri-c23041, cepharanthine, abemaciclib, osimertinib, trimipramine, colforsin, ingenol, and clofazimine have also been considered for in vitro evaluation of their *SARS-CoV-2* entry inhibition activities [27].

4.2 Virus genome replication

Generally, the virus replication directly affects the viral burden and symptom severity in viral infections. Therefore, targeting the key molecules in the *SARS-CoV-2* replication has been highly regarded for drug discovery against COVID-19. Previous studies confirmed that 3-chymotrypsin-like cysteine protease (3CLpro), papain-like protease (PLpro), RNA-dependent RNA polymerase (RdRp), and NSPs involved in the formation of double-membrane vesicles (DMVs) are vital for the replication of *SARS-CoV-2* [28]. Among the mentioned proteins, the 3CLpro is highly regarded as an attractive target for drug development against *SARS-CoV-2* because of its key role in the viral life cycle alongside the absence of closely related homologs in humans. Subsequently, to date, several

Name	EC50 (ng/ml)	References
BD-368-2	15	[12]
CB6	36	[13]
H4	896	[19]
P2B-2F6	410	[20]
B38	177	[19]
COV2-2196	15	[21]
COV2-2130	107	[21]
COV2-2165	332	[21]
CC12.1	22	[22]
C121	1.64	[23]

Table 2.
List of some neutralizing monoclonal antibodies against SARS-CoV-2 S1.

efforts have been made to identify the effective *SARS-CoV-2* 3CLpro inhibitors. The 3CLpro inhibitors are mostly categorized into peptidic and small molecules. Up to now, the efficacies of several 3CLpro peptide inhibitors such as N3, 13b, GC373, and GC376 have been validated. Moreover, some small molecules such as disulfiram, carmofur, ebselen, and tideglusib are known to inhibit 3CLpro from *SARS-CoV-2* [27, 29].

5. Current in use anti-COVID-19 treatments

Unfortunately, to date, there is no specific anti-COVID-19 drug. However, the results of some studies suggested that other anti-viral medicines could be repurposed as effective anti-COVID-19 drugs. Remdesivir, an FDA-approved repurposed antiviral drug, is only in used approved anti-viral therapy against COVID-19 [30]. However, other anti-viral and non-antiviral drugs have also been used for studying their anti-COVID-19 activities. Hydroxychloroquine, an anti-malaria drug with polymerase inhibitory activity, was the first repurposed drug against COVID-19, which was supported by some in vitro effectiveness evidence. However, further clinical trials indicate that there is no association between hydroxychloroquine administration and reduction in the death rate due to COVID-19. Kaletra (a brand name of lopinavir/ritonavir complex) is an approved anti-human immunodeficiency virus (HIV) protease inhibitor, which empirically evaluated for 3CLpro inhibitory activities. Despite, promising in vitro results, clinical trials have not confirmed the significant efficacy of Kaletra in individuals hospitalized with COVID-19. Favipiravir, a purine nucleic acid analog, is another anti-viral drug that is repurposed against mild to moderate COVID-19. The results of clinical trials suggest that Favipiravir has no significant beneficial effect on the mortality rate in patients with COVID-19. Additionally, some other drugs such as colchicine, oseltamivir, ivermectin, tocilizumab, nafamostat, camostat, famotidine, umifenovir nitazoxanide are under evaluation for investigating their probable anti-COVID-19 activities [31–33].

6. Computational drug repositioning

Because of the costly, time-consuming, and complexity of De novo drug discovery, until now all proposed anti-COVID-19 drug candidates are repurposed drugs. Drug repurposing also known as drug re-tasking is a procedure of recognizing new therapeutic application(s) for previously approved, failed, investigational, and or already marketed drugs. Naturally, the drug-repurposing process is based on two fundamental principles including interdependence between different diseases and the confounding nature of drugs. Therefore, drug-repositioning approaches could be categorized into drug-based and disease-based strategies.

The drug-based strategies are vastly based on drug-related data and are used for better understanding the role of pharmacological properties and defining the possibility of defining new pharmaceutical capabilities. Despite the advantages of experimental drug repositioning, the fact that it was time consuming still remained as the main limitation for drug discovery, especially in a pandemic condition. Furthermore, conventional methods use small datasets and biological networks, which may lead to unreliable discoveries.

Nowadays, different computational methods have been introduced that can accelerate the drug-repositioning process [27]. In the next sections, the most common computational approaches for drug repositioning are propounded.

6.1 Molecular target identification and validation in the drug-repositioning process

In a drug discovery project, target identification and validation are key steps that directly affect drug efficacy, as well as probable side effect(s). Theoretically, a drug target molecule can be selected among a wide range of biological entities including proteins, genes, and RNAs. However, an ideal drug target molecule should be drug accessible, efficacious, safe, and meet clinical and commercial requirements [4]. Target identification can be performed by different tools such as analysis of gene modifications, protein overexpression, signaling pathways, protein interactions, and recent bioinformatics evaluations. Regarding antiviral drug discovery, different targets such as envelop proteins, S-adenosyl-L-homocysteine hydrolase, orotidine 5′-phosphate decarboxylase, cytidine triphosphate synthetase, inosine monophosphate dehydrogenase, and DNA/RNA polymerase have been investigated for discovering effective antiviral drugs [34–37]. The identified target molecules can be validated by knocking in/down/out the genes, monoclonal antibodies, and chemical genomics [4, 38]. As mentioned, recently bioinformatics methods, such as ligand-based interaction fingerprint (LIFt), protein-ligand interaction fingerprints (PLIF), and network-based drug discovery, have successfully been used for drug target identification [39].

6.2 Data mining

There are now a large number of diseases- and drugs-linked information such as gene sequences, protein–protein interactions, and drug–protein interactions with increasing rapid growth, which needs effective approaches to quick access and analysis of hidden information. Commonly, text mining is the most applicable method in the majority of data mining–related studies. In the field of computational drug repurposing, text mining has been used to find the gene, drug, and diseases-related data and then categorize the relevant entities. Regarding drug repurposing, text mining has successfully been used in several studies [40, 41]. Brown et al. suggested an online text-mining server with the ability to drug clustering based on the similarity of their physicochemical properties [42]. A text mining-based tool was also introduced by Leaman et al. for identifying disease-related information mentioned in the literature [43]. In another study, Papanikolaou et al. used text mining to recognize biological entities in the Drug Bank database. The retrieved data were then clustered by different algorithms and used for obtaining novel drug–drug relations [44].

6.3 Machine learning (ML)

Machine learning, a crucial subset of artificial intelligence (AI), has been combined into many fields, such as data generation and analytics. Related to drug discovery, ML algorithms may participate in target and lead discovery as well as develop quantitative structure–activity relationships. Briefly, in machine learning-based drug repositioning, different algorithms, such as artificial neural networks (ANNs), support vector machines (SVMs), and random forest (RF), were trained by numerical forms of different features of drugs, diseases, genes, and so on. The trained algorithms can then predict the drug ability of unknown compounds [45]. In this regard, Gottlieb et al. used drug–drug and disease–disease similarity events as grouping features for training a logistic regression classifier and prediction of drug-disease associations [46]. Similarly, Napolitano et al. introduced a SVM model trained by drug-related similarities with the ability to forecast the therapeutic class of United States Food and Drug Administration (FDA)-approved compounds [47]. Aliper et al. introduced a fully connected deep neural network algorithm trained

by gene expression signatures for predicting therapeutic potentials and new drug suggestions [48].

6.4 Network analysis

Biological networks, an outstanding way of modeling biological entities and their interactions, can supply significant insight into the mechanism action of drugs and drug targets and symptoms of diseases. The models can be used to determine informative associations between genes, chemicals, proteins, phenotypes, and any other biological entities by statistical analysis, computational models, and leveraging graph theory concepts. Based on the data sources, network analysis can be classified into metabolic networks, protein–protein interaction networks, drug–drug interaction networks, drug-side effect association networks, disease–disease interaction networks, and gene regulatory networks. Consequently, bionetworks and their analysis can be used to identify potential therapeutic agents and drug repositioning [49–51].

6.5 Molecular docking

Studying the ligand-protein interactions at the molecular level has a crucial role in pharmaceutical research. Therefore, the scientific community focused on the exploration of the binding phenomenon over the years. Accordingly, some theories, such as lock and key hypothesis, induced-fit theory, and conformational selection were introduced for the interpretation of ligand–protein interactions [52]. Historically, the refinement of a complex structure by optimization of the separation between the partners was the first description of the docking term in 1970. Molecular docking was first being developed in 1980 to predict the best matching binding mode and the molecular interactions of a ligand to a macromolecular partner through the generation of a number of probable orientations of the ligand inside the protein cavity. The method comprises two interrelated steps including orientations sampling and a scoring function, which are responsible for reproducing experimental binding mode and ranking of prepared complexes [52, 53]. Molecular docking can classify into rigid, semi-flexible, and flexible types, according to the degrees of flexibility of the ligand and receptor. In the rigid docking-like to lock-key theory, both ligand and protein are considered rigid entities and hence, there is no internal degree of freedom. Semi-flexible docking is a molecular docking simulation with flexible ligand and rigid receptors. Thus, all degrees of freedom of ligand are explored. Recently, several online and standalone software such as AutoDock, AutoDock Vina, Molegro Virtual Docker, Gold, Surflex-Dock, GLIDE, FlexX, DOCK, FRED, and so on, have been developed for computing different types of molecular docking. Most available software for molecular docking uses flexible ligands and several are trying to model flexible receptor proteins. In recent years, with promising advancements in optimization and the development of new molecular docking algorithms, numerous publications have been planned for comparing the performance of different molecular docking tools. However, it should be stressed that comparison between molecular docking methods is problematic, due to the dependance on docking performance with classes of the subjected targets. The ability of molecular docking methods to reveal the possibility of enzymatic reactions is a compelling reason for various applications related to computational drug design and repurposing, hit identification, lead optimization, binding site prediction, mechanisms of enzymatic reactions, and protein engineering [54–56]. Since the emergence of COVID-19, several molecular docking-based studies [57–62] have been planned to introduce effective anti-COVID-19 drugs by means of drug repositioning. In **Figure 1**, the main steps of a molecular docking-based drug repurposing study are represented.

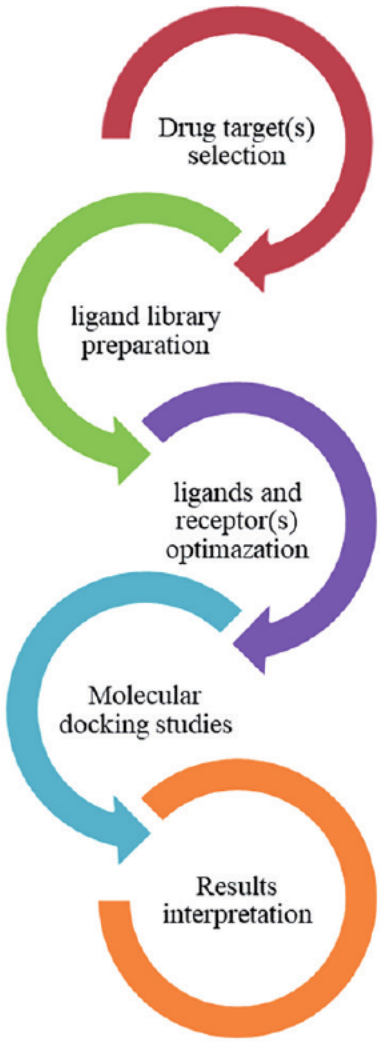

Figure 1.
Schematic representation of the main steps of a molecular docking-based drug repositioning process. Target identification, ligand preparation, and results interpretation are the three main steps.

6.5.1 Recent projects, challenges, and future prospects in molecular docking-based drug repositioning against COVID-19

As a popular bioinformatics method, recently several types of research have been conducted to reposition approved drugs against COVID-19 by means of molecular docking. Despite similar aspects and methodology, the used software, subjected target and ligands can affect the outputs of molecular docking-based drug repositioning [54, 63]. In **Table 3**, some recently published works associated with molecular docking-based drug repurposing are presented. Based on our best knowledge, *SARS-CoV-2* main protease is the most popular target for drug discovery research due to the absence of closely related homologs in humans. Additionally, some host cell proteins such as Angiotensin-converting enzyme 2 (ACE2), Transmembrane Serine Protease 2 (TMPRSS2), Furin, Cathepsin L, Adaptor-Associated Kinase 1 (AAK1), and Two-Pore Channel (TPC2) have also been regarded for drug discovery against COVID-19. However, due to probable side effects, drug repurposing based on host cell targets received less attention.

Subjected target	ligands	Proposed drug or ligand	References
Mpro	FDA-approved drugs	binifibrate and bamifylline	[64]
Mpro	4384-approved drugs	Daunorubicin and eight other compounds	[65]
Mpro	6218-approved drugs	Emodin and blonanserin	[66]
RBD, NSP 10, NSP 16, Mpro, and RdRp	Brazilian Public Health System-approved drugs	penciclovir, ribavirin, and zanamivir	[67]
Mpro	Drug Bank database	levothyroxine, amobarbital and ABP-700	[68]
spike glycoprotein	FDA-approved drugs	Conivaptan and Trosec	[14]
spike glycoprotein	Plant secondary metabolites	Dicaffeoylquinic acid	[15]
Mpro	FDA-approved antiviral drugs	Lopinavir-Ritonavir, Tipranavir, and Raltegravir	[16]
papain like protease	Plant secondary metabolites	I-Asarinin	[17]
Mpro	superDRUG2 database	Binifibrate and Bamifylline	[18]
Mpro	Plant secondary metabolites	ursolic acid, carvacrol and oleanolic acid	[24]
RdRp	FDA-approved anti-viral drugs	remdesivir, ribavirin, sofosbuvir and galidesivir	[25]
Mpro	FDA approved drugs	remdesivir and glycyrrhizin	[26]
Mpro and RdRp	Plant secondary metabolites	cryptomisrine, cryptospirolepine, cryptoquindoline, and biscryptolepine	[27]

The SARS-CoV-2 main protease is the most considered target for drug discovery.

Table 3.
Recently published molecular docking-based drug repositioning research for introducing novel drugs against COVID-19.

Regarding the subjected ligands evaluation of their anti-COVID-19 potentials, there are several choices, including approved standard drugs, approved natural products, plant secondary metabolites, and under investigation drugs. Due to the time-consuming approval drug process as well as unexpected side effects, drug repurposing based on the approved drugs database is highly recommended [69, 70]. Despite the advantages of in silico drug repositioning against COVID-19, due to differences between natural drug-target micro-environments and drug-target simulations, the discrepancy between the laboratory results and the simulation outputs is expected. Therefore, a recently mixed approach, which is the combination of computational and empirical methods is proposed to fast and accurate drug repositioning [5].

6.5.2 A case study: repurposing FDA-approved antiviral protease inhibitors as SARS-CoV-2 3CLpro inhibitors

As mentioned in Section 3.2, due to the important role in the viral life cycle alongside the absence of closely related homologs in humans, the 3CLpro is considered a proper target for discovering effective antiviral drugs against *SARS-CoV-2*. Therefore, here a molecular docking-based drug-repurposing process was planned to screen the 3CLpro inhibitor(s) among the standard antiviral protease blockers.

6.5.2.1 Retrieval and preparation of ligands and receptor

A small molecule–protein molecular docking study is based on the prediction of probable interactions between the ligand and its receptor. Obtaining the three-dimensional structures of both the ligand and receptor is the first vital step for performing a molecular docking process. Therefore, the raw three structures of a set of FDA-approved antiviral protease inhibitors, as well as 3CLpro from *SARS-CoV-2*, were retrieved from the drug bank database (https://go.drugbank.com/) and protein data bank (https://go.drugbank.com/) respectively. The subjected drugs (**Table 4**) were obtained in the sdf format, and their raw structures were further prepared by adding polar hydrogens, computing Gastieger charge, detecting the root atom, setting the torsion, and the number of torsions. Furthermore, the structure of the 3CLpro was also optimized by deleting water molecules and bound ligands, adding polar hydrogens and Kollman charge using the Python molecule viewer software.

6.5.2.2 Primary screening by blind docking method

Despite primary screening done by the blind docking method, several studies have been conducted to introduce effective 3CLpro inhibitors. However, to date, binding pockets and key amino acids in the enzyme catalytic activity are not well known. Therefore, as primary screening, the blind docking processes through Molegro Virtual Docker 6.0 software were performed between the standard drugs and the 3CLpro to determine the key amino acid(s). In blind molecular docking, the whole surface of a subjected receptor is considered for evaluation of probable interactions with the ligand.

6.5.2.3 Targeted molecular docking

After determining the total affinities of the standard drugs to the 3CLpro as well as more reactive amino acids, targeted molecular docking studies were conducted between the receptor the three top-scoring docked ligands in a grid box, which covers the key amino acid(s) by Autodock 4.2.6 software.

6.5.2.4 Results

The results of the primary screening are presented in **Table 5**. The results demonstrated that Amprenavir, Tipranavir, and Fosamprenavir had a higher

Approved drug	Chemical formula	Accession number
Darunavir	C27H37N3O7S	DB01264
Tipranavir	C31H33F3N2O5S	DB00932
Atazanavir	C38H52N6O7	DB01072
Amprenavir	C25H35N3O6S	DB00701
Fosamprenavir	C25H36N3O9PS	DB01319
Nelfinavir	C32H45N3O4S	DB00220
Ritonavir	C37H48N6O5S2	DB00503
Indinavir	C36H47N5O4	DB00224
Saquinavir	C38H50N6O5	DB01232

Table 4.
Chemical formula and drug bank accession number of nine FDA-approved antiviral protease inhibitors subjected for repurposing against SARS-CoV-2.

Drug	Moldock score (kcal/mol)	Key amino acids
Darunavir	−110.402	THR190,ARG188, ASN142,TYR54,GLN189, ASP187
Tipranavir	−158.307	THR26, ASN142,GLN189, ARG188,GLU166
Atazanavir	−77.870	GLN189,TYR154
Amprenavir	−160.384	TYR154,GLN127,VAL303
Fosamprenavir	−146.601	CYS145,GLN189,SER144,GLY143,LEU141,HIS41,MET165,GLU166
Nelfinavir	−70.32	CYS145,GLN189
Ritonavir	−101.440	GLN189,THR24,CYS145
Indinavir	−98.704	GLN189,GLY143,THR26
Saquinavir	−100.442	GLU166,GLN189,THR26

Table 5.
The results of blind molecular docking between the standard antiviral protease inhibitors and the 3CLpro from SARS-CoV-2. Amprenavir, Tipranavir, and Tipranavir showed high binding affinity to 3CLpro.

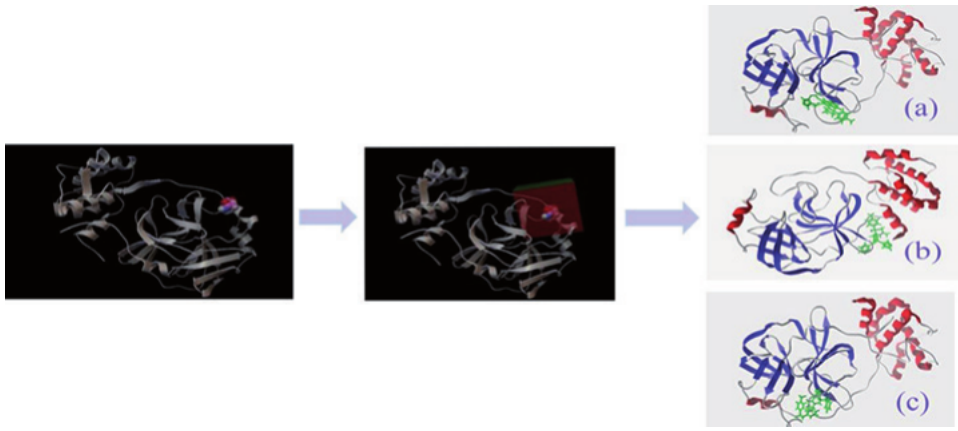

Figure 2.
Graphical representation of the targeted molecular docking between the 3CLpro from SARS-CoV-2 and (a) Fosamprenavir, (b) Amprenavir, and (c) Tipranavir. Fosamprenavir showed the most binding affinity in the subjected docking grid box followed by Amprenavir and Tipranavir respectively.

binding affinity to the 3CLpro than the other tested viral protease inhibitors with Moldock scores of −160.384, −158.307, and −146.601 respectively. Furthermore, it was clear that GLN 189 is a key amino acid in the 3CLpro interactions with different proteases. Therefore, a targeted molecular docking between the three top-scoring standard protease inhibitors (Amprenavir, Tipranavir, and Fosamprenavir) were also performed in a grid box with the center of GLN189. As depicted in **Figure 2**, the subjected standard drugs also showed high affinity to the 3CLpro with binding energies of −5.3, −5.1, and −6.2 kcal/mol respectively. Subsequently, due to the high affinity of Fosamprenavir to the 3CLpro, this antiviral protease inhibitor could be considered for further in silico, in vitro, and in vivo evaluation to develop as a repurposed anti *SARS-CoV-2* treatment.

7. Conclusion

To date, the only approved anti-COVID-19 treatment is a repurposed antiviral drug (Remdesivir). Hence, drug repurposing might be an effective approach for accelerating drug discovery against COVID-19. Computational drug repositioning offers a noteworthy reduction in time and costs of new drug development and increases success rates in comparison to traditional methods. Therefore, to date, different computational methods such as data mining, machine learning, network analysis, and molecular docking have successfully been used for drug repurposing.

Molecular docking is a popular bioinformatics method that recently has been highly regarded for studying the drug ability of biological entities, protein-ligand interactions, mechanism action of drug candidates, and drug repositioning. Retrieval drug candidates from standard databases or previous reports, lead and target optimization, running the molecular docking process, and results analysis are the main steps in molecular docking-based drug repositioning. The binding affinity of a drug candidate to key amino acid(s) of the identified target molecule can be considered a decision factor in the drug repositioning process.

Despite the advantages of computational drug repositioning, studying drug-target interactions by in silico methods is still far from reality.

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Drug Repurposing Techniques in Viral Diseases

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Abstract

Since the advent of the twentieth century, several severe virus outbreaks have occurred—H1N1 (1918), H2N2 (1957), H3N2 (1968), H1N1 (2009) and recently COVID-19 (2019)—all of which have posed serious challenges to public health. Therefore, rapid identification of efficacious antiviral medications is of ongoing paramount importance in combating such outbreaks. Due to the long cycle of drug development, not only in the development of a "safe" medication but also in mandated and extensive (pre)clinical trials before a drug can be safely licensed for use, it is difficult to access effective and safe novel antivirals. This is of particular importance in addressing infectious disease in appropriately short period of time to limit stress to ever more interlinked societal infrastructures; including interruptions to economic activity, supply routes as well as the immediate impact on health care. Screening approved drugs or drug candidates for antiviral activity to address emergent diseases (i.e. repurposing) provides an elegant and effective strategy to circumvent this problem. As such treatments (in the main) have already received approval for their use in humans, many of their limitations and contraindications are well known, although efficacy against new diseases must be shown in appropriate laboratory trials and clinical studies. A clear in this approach in the case of antivirals is the "relative" simplicity and a high degree of conservation of the molecular mechanisms that support viral replication—which improves the chances for a functional antiviral to inhibit replication in a related viral species. However, recent experiences have shown that while repurposing has the potential to identify such cases, great care must be taken to ensure a rigourous scientific underpinning for repurposing proposals. Here, we present a brief explanation of drug repurposing and its approaches, followed by an overview of recent viral outbreaks and associated drug development. We show how drug repurposing and combination approaches have been used in viral infectious diseases, highlighting successful cases. Special emphasis has been placed on the recent COVID-19 outbreak, and its molecular mechanisms and the role repurposing can/has play(ed) in the discovery of a treatment.

Keywords: viral infectious disease, COVID-19, drug development, drug repurposing, drug repurposing strategies, applications

1. Introduction

The development of a new drug is an extensive, intricate, highly risky and expensive process. According to the study of 12,728 transitions over the last decade

(2011–2020), the success rates of clinical drug development were 52.0% (Phase I), 28.9% (Phase II) and 57.8% (Phase III) separately [1]. Most of the candidates failed in the early drug development process for reasons of efficacy and safety concerns [2]. This causes a huge cost in drug development, ranging from tens of millions to billions. In addition, it typically takes at least 10 years from initial laboratory evaluation for a new drug to be approved. All these factors lead to low output disproportionately to the high input and make it challenging for the pharmaceutical industry to respond to emergent infectious diseases within a time frame that is able to impact the course of an outbreak. As a result, the development of vaccine candidates will likely remain to be the optimal mechanism to address outbreaks in the immediate future. Notwithstanding the extraordinary developments in vaccine technology—exemplified by recent events [3, 4]—there still remains a need for therapeutics as a sufficiently successful virus will rapidly become pan/endemic, creating a constant need for treatment of those unfortunate enough to not have access to a vaccine due to socioeconomic, age or immunostatus issues. Indeed, in the case of a pandemic, there is likely to be constant strain on health systems to treat patients, which will significantly enhanced in the absence of an effective therapy as it will instead rely heavily on patient support technologies such as ventilation or oxygen therapy.

A potential solution to this conundrum is the examination of already proposed (ideally approved) medications—repurposing—to assess their potential in the treatment of an emergent diseases. Drug repurposing as a strategy to identify potential new indication areas of approved/old drugs has many advantages. Firstly, low risk. Most repurposed drugs have at least been tested in early clinical trials. Hence, the failure rate of repurposing candidates caused by safety is very low. Secondly, low cost. Most old or approved drugs have clear safety, pharmacokinetics and pharmacodynamics data, which reduces the studies that need to be performed before the drugs extension to a novel indication. Thirdly, a higher success rate. The drugs used for repurposing are enriched from previous studies. This means less promising compounds are filtered out, allowing for a higher success rate [5]. Finally, while the molecular complexity of the protein targets of diseases is extremely broad, areas of essential molecular function can be identified as conserved in many diseases. For example, tyrosine kinase activity is a frequent target for the development of cancers from highly diverse tissues. A common feature of all kinases is the presence of an ATP-binding site, which has resulted in a large number of targeted cancer therapies (tyrosine kinase inhibitors, TKIs) which have a strong resemblance to ATP on the molecular level. This has further resulted in the clinical testing of TKIs for cancers distinct to those for which they were developed, as the evolutionary pressure to retain a function ATP-binding site provides a precondition for potential TKI cross-reactivity [6].

In the past decades, the successful application of drug repurposing shows a promising direction for drug development. For example, Thalidomide was firstly synthesized by Ciba in 1953 and came on to the market to relieve morning sickness in 1957. In 1961, Thalidomide was taken off the market due to the severe teratogenic effect on the developing fetus. In the following years, an Israeli researcher found that thalidomide could be used as a treatment against autoimmune diseases. In 1998 it was approved by FDA for the repurposed use in the treatment of ENL [7–9]. In this chapter, we present an overview of the benefits and drawbacks of drug repurposing in viral disease, including approaches, applications and outlook.

2. Drug repurposing

Drug repurposing is the process of identifying new indications and uses for approved/existing drugs [10]. It mostly involves approved drugs or compounds

under study, which have clear pharmacokinetics and pharmacodynamics that provide data on metabolic stability, tissue distribution and clearance rates. In the past decades, a large quantity of new molecular entity drugs was approved or studied, meaning that not only the ~2000 FDA approved compounds can be screened [11, 12], but also a much larger potential library of compounds that have been developed to have appropriate physico-chemical properties for their use as drugs, but that may have failed clinical trials due to lack of action in their original disease class. This, perhaps, is the key benefit of repurposing—as all potential repurposing candidates possess "drug-like" properties. However, these properties should be borne in mind by the researcher as they strongly impact the potential of repurposed compounds in clinical use.

The classic description of "drug-like" properties has grown significantly since the original introduction of the "Rule of 5" by Lipinski (Ro5) [13]. This initial classification arose from the observation that successful drugs shared common properties: low molecular weight (Mw), a relative scarcity of potential electrostatic interactions (H-bond donors and acceptors) and a partition coefficient (logP) that indicated the molecules would be able to passively diffuse across cell membranes. While, the most important descriptors remain unchanged a summary of Lipinski's and others rules is provided in **Table 1** [13–17]. As repurposing candidates will be drawn from compounds that are likely to be enriched for these properties and therefore the potential route of administration for the repurposed disease should be compatible with these. For instance, compounds that are a good fit to Ro5 would be relatively poorly applied to diseases for which administration would be via inhalation [18]. Thus, the availability of this pharmacological information and likely route of administration is of key importance in deciding on which compounds should be assessed for repurposing.

In summary, these studies provide a wealth of information about the clinical application and mechanism of action, aiding the rapid development of the drug repurposing. When compared with *de novo* drug discovery, drug repurposing accelerates the development process and significantly reduces development risk [19].

2.1 Drug repurposing approaches

At its broadest level drug repurposing approaches can be divided into two geneal types: computer-based and experimental techniques [20, 21]. Within both these approaches there are three main angles of attack, drug-centric, target-centric and disease-centric methods [22]. As indicated by its name, drug-centric approaches start from the point of view of a drug, with the aim to find efficacy against diseases other than the initial indication. In the case of a disease-centric approach the disease is the focus, with the purpose being to identify and repurpose a drug

RO5	RO3	Ghose rules	Veber's Rules	MDDR-like rules
MW ≤ 500	MW < 300 Da	160 ≤ MW ≤ 480	NRTB < 10	RNG ≥ 3
HBD ≤ 5	HBD ≤ 3	−0.4 ≤ logP ≤5.6	PSA < 140 Å	RGB ≥ 18
HBA ≤ 10	HBA ≤ 3	30 ≤ AMR ≤ 130		NRTB ≥6
LogP ≤ 5	cLogP ≤3	20 ≤ NA ≤ 70		
	NRTB ≤3			

Abbreviations: MW, molecular weight; HBD, H-bond donor; HBA, H-bond acceptor; logP, octanol-water partition coefficient; clogP, calculated octanol-water partition coefficient; AMR, molar refractivity; PSA, total polar surface area; RGB, the number of rigid bond; HB, hydrogen bond; NAT, the number of atoms; NRTB, number of rotatable bonds; RNG, number of rings.

Table 1.
Summary of 'druglikeness' rules applied in drug development.

specifically against that disease. Target-centric methods utilize drugs that bind to well-characterized targets that are known to be, or at least suspected to be, involved in other diseases besides the drugs' original indication. What they have in common is that at the core these strategies often employ similarity assessment to identify drugs that can potentially be repurposed.

2.1.1 Computer-based approaches

Traditional drug repurposing often relies on the in vitro/in vivo identification of active drugs or alternative targets. Whilst this can provide promising compounds with reliable, desired activity, it can be expensive, involves physical access to the drug libraries and requires setup and optimization of the assays [23]. With the rapid development of bioinformatics and the accumulation of vast amounts of experimental data, the development of drug repurposing, especially the initial stage, has moved from traditional biological experiments towards an increasing diversity of computational screening approaches, partially due to the lower cost and lower barrier to entry [24].

2.1.1.1 Virtual screening

Virtual screening is an essential computational approach in drug discovery, and particularly in drug repurposing. It involves the use of computer programs to evaluate compound libraries on a specified criterion, usually similarity or calculated binding energy. Virtual screening can be classified into two categories: ligand-based and structure-based virtual screening [25, 26].

Ligand-based screening focuses on analyzing the structure-activity information of known active ligands against a certain indication to identify other potentially effective drugs. This analysis relies on similarity in the form of pharmacophores and geometric shape which can be informed by structural knowledge of the ligand-target complex to identify key pharmacophores or without structural information, relying on the structure-activity relationship information from experimental approaches to identify the pharmacophores [27]. Pharmacophores are the chemical moieties of drugs that play essential roles in the interaction with their targets. Pharmacophore features—including features such as hydrogen bond donors, hydrogen bonds acceptors, charge groups, aromatic rings and hydrophobic centroids are then identified together with their spatial characteristics and mapped into a string [28]. This string can serve as a fingerprint, which can be used for easy similarity matching between different drugs, potentially identifying drugs that are also active against the disease.

This approach can be successful for small molecules as they are relatively simple molecules from a chemical perspective. Their pharmacophoric features are limited and usually rely on a few strong, deeply buried interactions with the target, making it easy to map and identify drugs with similar characteristics [29]. In contrast, biologics, such as peptides and antibodies, are far less suitable to these techniques as their method of actions typically dependent on mimicking protein-protein interactions, which are characterized by large, flat interaction surfaces [30, 31]. This makes them highly specific for their target, allowing for targeted therapies with typically less side-effects, but that specificity also prevents them from being repurposed for a different target.

In contrast, structure-based virtual screening uses the three-dimensional structure of a target of therapeutic interest [32, 33], which is screened against a virtual library of approved drugs in order to identify those that show interactions with this novel target. Drugs are docked against the target and interaction analysis

is performed based on binding energy and binding geometry. Many different docking software packages have been developed with the key differences being in the docking methodologies and the scoring functions used to rank the drugs [34]. Classical scoring functions usually rely on experimental data or prior information to rank the drugs. However, these have been consistently getting outperformed by machine learning based scoring functions, especially when specific target data is available to train on [35, 36].

Developments in computational power have made virtual screening approaches highly accessible to labs all over the world as they do not require nearly the amount of financial resources compared to wet-lab experiments. In addition, due to the speed at which the screenings can be performed nowadays, huge libraries containing 100's of millions of compounds can be screened against a target rapidly massively increasing the chemical space explored [37]. Though these advances are very useful in early drug discovery, where it can be used to screen fragment and compound libraries that cover a diversity in chemical space, they are less impactful when it comes to drug repurposing since the amount of approved drugs is limited and does not comprise wide chemical space. A downside of structure-based screening is the need for the actual structural information, which can be difficult to obtain for novel targets. Fortunately the number of entries available in the PDB has been growing at an exceptional rate, more than doubling in the last decade [38], meaning more and more targets have structural information available. In addition, the recent achievements of AlphaFold [39], including the prediction of the 3-dimensional structures of the entire human proteome, might alleviate this issue [40]. Overall this still has a positive impact on drug repurposing strategies as the more structural information is available the better scoring functions can be become, aiding both ligand-based and structure-based methods in identifying drugs that can be repurposed.

2.1.1.2 Machine learning approaches

Machine learning is an overarching term used to describe diverse algorithms that use data sets to perform intelligent predictions [41]. The algorithms can be trained on large datasets to identify patterns and interactions. The trained algorithm can then be applied to novel data to identify or predict outcomes or interactions.

Computer based drug repurposing techniques utilizing machine learning have been gaining a lot of traction due to a large increase in available omics data in a variety of databases and the development of sophisticated algorithms that can utilize this data [42–44]. It is carried out using computational biology, bioinformatics and database tools, which allows for economical and high efficiency drug discovery [45]. Machine learning techniques used for drug repurposing include: k-nearest neighbor algorithms, decision tree, random forest, artificial neural networks, k-means clustering and principal component analysis [20, 46, 47].

In recent years researchers have not been able to keep up with the amount of information being generated by omics experiments, creating a need for different data analysis methods. Where previously they would manually comb through the data looking for patterns and connections, there has been a shift towards big data analysis utilizing machine learning approaches, which have shown several specific applications in drug repurposing [48].

Signature matching is an approach where complex patterns and profiles—signatures—are generated for diseases and drugs by machine learning algorithms from large omics datasets. By looking for negative correlations between differential

signatures resulting from diseases and from drug treatments, drugs can be identified that can serve as treatments for those diseases outside of their original indication [5, 20]. Simultaneously, drug signatures can also be compared with the signatures of structurally dissimilar drugs, with the idea being that if drugs show a similar signature they can share a therapeutic application irrespective of chemical similarity. For both these applications there is an alternative signature that can be compared, the clinical phenotype signature. Even though some diseases or drugs might show little to no similarities in direct transcriptomic, metabolomics or proteomic patterns, they could still have similar clinical phenotypic outcomes, which can also allow for the identification of repurposing uses of drugs [49].

Another use of signature matching is in finding similar chemical features of drugs and mapping a network based on shared features. This allows for the identification of drugs that may potentially be repurposed—as similarity in pharmacophores tends to correlate with a similarity in biological activity.

Related to signature-based methods, application of genome-wide association studies (GWAS) have also shown to be valuable within the field of drug repurposing [50]. GWAS data can be analyzed using machine learning approaches to identify interaction and association patterns of genes linked to diseases [51]. Genes identified by GWAS to associate with a disease tend to be enriched with druggable targets. By cross-referencing the disease enriched genes with databases containing drug-target information drugs can be found that inhibit specific genes that are involved in other indications but also seemingly play a role in the GWAS investigated disease, potentially being able to reuse that drug. In addition if a gene is shown to be associated with a disease it could become a novel drug target, which can be screened against using approved drug libraries.

Even though GWAS identified genes can be associated with a disease that does not mean that the target is druggable. Pathway mapping could be a potential tool to leverage the information gained with GWAS and expand upon it [52]. By analyzing the pathways or protein interaction networks up and/or downstream of the GWAS identified genes, other, previously elusive, proteins can be identified that could play a role in disease progression. This can either yield new drug targets or repurposing opportunities of drugs that already inhibit the elucidated target. For example, pathway analysis was performed on data sets containing gene expression data from human hosts infected with many different respiratory viruses. This identified 67 conserved biological pathways that could play an important role in respiratory viral infections. Comparing these pathways to a drug-target database resulted in drugs like pranlukast and amrinone, drugs with a different indication, that could potential be used in treating viral infections [53].

2.1.2 Experiment-based approaches

Empirical evidence is still highest order of evidence and remains the golden standard for drug screening, including drug repurposing. Since experimental assays provide the most immediate evidence of drug activity [51] they are not only used to discover potential repurposing candidates from libraries but they are also essential in validating hits from computational approaches.

Inhibition assays can serve to identify target-specific drug efficacy, including inhibition constants. Binding assays are very powerful as they can also provide binding constant information [54]. Immediate use can be made of the identified binding drug that might not be highly specific or effective but it could serve as a temporary stop gap in emergency situations (like pandemics). Whilst the repurposed drug is being used as a sort of band aid, drug development can be undertaken in parallel, using the drug as the starting point. Rapid SAR approaches can then be utilized to improve the drug binding

and efficacy [55]. The fact that the resulting drug would ideally be quite similar to the approved drug could lead to accelerated approval processes.

2.1.2.1 Binding assays

Binding assays aim to detect the interaction(s) between two (bio)molecules, such as protein-protein, peptide-protein, nucleic acid-protein, small molecule-protein, or small molecule-nucleic acid and ideally also evaluate the degree of the interaction [56]. These assays can be used in two ways, in screening approaches to qualitatively identify hits that interact with the target and in a quantitative way to characterize the binding affinity.

There are many examples of different types of qualitative assays that have been used in drug repurposing approaches. Among the most common are immobilization or affinity chromatography, where either the target or the drug are immobilized on a matrix or column followed by exposure to a drug library or potential binding targets [57]. The complexes that have formed can then be eluted and identified using analytic methods. DNA-encoded libraries encompassing wide chemical space have been used in such approaches. After eluting complexes binding compounds are identified by sequencing the DNA-barcode attached to the binding compound. This technique can also be applied to approved drug libraries [58].

The aforementioned assays are aimed at screening large libraries for hits. However, obtaining detailed binding information such as dissociation constants (K_D), is crucial in the identification and development of potent drugs. Several biophysical techniques are available to quantify these interactions. Microscale Thermophoresis (MST) can be used to measure binding affinity by detecting changes in molecular motion in a temperature gradient in the presence and absence of different compound concentrations [59]. Differential scanning fluorimetry (DSF) can be used to measure protein unfolding temperature by monitoring in fluorescence of a probe that binds to hydrophobic moieties in a denaturing temperature gradient. Upon binding of drugs to the protein it can stabilize the complex, leading to a shift in unfolding temperature. By using a range of drug concentrations and measuring the effect on the thermal shift the K_D can be calculated [60]. Surface plasmon resonance (SPR) is a technique in which the target or drug of interest is immobilized on a thin metal film. A light source is aimed at the other side of the film and the surface Plasmon resonance angle is detected. When a drug or target binds to the immobilized partner the local mass at the sensor surface changes, causing a shift in the angle of reflection proportional to the mass. By measuring these changes in the presence and absence of drug or target, association and dissociation constants can be determined [61]. Isothermal titration calorimetry (ITC), one of the golden standards in K_D determination, can directly measure all binding parameters by measuring heat transfer. When binding of drug to target occurs, enthalpy changes (heat absorbed or released) of the system can be measured by a highly sensitive calorimeter. By titrating ligand against the target of interest the Kd, stoichiometry, enthalpy and entropy can be directly measured in the native states of the binding partners since no modifications are required [62].

2.1.2.2 Phenotypic screening

Where binding assays are typically focused on identifying target-drug interactions, phenotypic screening takes a more disease-centric approach. Phenotypic assays aim to identify compounds that show effects on disease-relevant outcomes [63]. These are usually performed on cell lines or organelles engineered to function as disease models. Since the assay is target agnostic less, or no, information about specific targets is obtained. However, the fact that it is agnostic also means that there are more potential

targets available within this complex environment, which could lead to the discovery of new targets that would otherwise be left unexplored [63]. There are also additional benefits to this approach in the context of drug repurposing. Since the assays are disease based and compounds are approved drugs or clinical candidates it means that if positive outcomes are obtained the drug already has positive properties and shown efficacy in more complex systems, which is beneficial to real world applications [64].

2.1.3 Side effect based or "serendipitous" drug repurposing

One of the most frequent reasons for drugs failing in (pre)clinical trials is the determination of a side effect that cannot be ignored. Most commonly this is determined to be a dangerous side effect that argues against further clinical investigation of the compound. However, one man's meat is another man's poison. These drugs with unwanted side effects can be given new indications through a drug repurposing strategy. Side effects-based drug repurposing links indications with clinical effect and is one of the common strategies employed for drug repurposing [65, 66]. A key example in this area is Sildenafil, which was originally entered into clinical trials as a drug to treat hypertension and angina [65, 67–70]. Unfortunately, Phase I clinical trials suggested that it had little effect on angina. However, use of Sildenafil causes a significant side effect: marked penile erections. This lead to the discovery that Sildenafil could be used as a treatment for erectile dysfunction (ED) [71]. In 1988, Sildenafil was approved by the FDA for the treatment of ED. Such repurposing approaches could be termed serendipitous repurposing, as the new indication area is revealed during clinical trials. As a result, such repurposing is relatively rare.

3. Drug repurposing in viral diseases

Over the last decades the world has seen multiple severe viral outbreaks resulting in millions of deaths. Among the deadliest were the Influenza pandemics such as H1N1 (1918), H2N2 (1957), H3N2 (1968) and H1N1 (2009). The HIV/AIDS epidemic that was first recognized in the 1980s and went global has also caused up to an estimated amount of 36 million deaths and is still ongoing. Besides the large, deadly pandemics there have been smaller but very impactful localized epidemics such as Dengue virus (DENV), Zika virus (ZIKV), Ebola virus (EBOV) and Middle East respiratory-syndrome corona virus (MERS-CoV) which pose serious challenges to public health. Most recently in 2019 there was an outbreak of Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which caused the COVID-19 pandemic, affecting nearly every country in the world with over 226 million reported cases to date [72]. Despite the advances in controlling viral pathogens that come with the widespread mass vaccination, there are no approved specific (effective) therapies for the treatment of most viral infections.

By exploring new targets and mechanisms, drug repurposing provides new indications for old drugs. The major time advantage of repurposing is that this approach allows repurposed drugs to quickly enter clinical trials, which is of significant importance in reacting to disease outbreaks, especially in the case of worldwide pandemics.

3.1 Drug repurposing for COVID-19

The outbreak of COVID-19, caused by SARS-CoV-2, has spread across the world. There is, as yet, no specific treatment for COVID-19 approved. Drug repurposing provides a fast and economical option for the identification of medications targeting SARS-CoV-2.

SARS-CoV-2 is a member of of the betacoronaviruses family. It is a single-stranded RNA virus, characterized by large crown-like spikes protruding on the viral surface and an unusually large RNA genome which encodes four main structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N) [62, 73, 74]. Like other coronaviruses, SARS-CoV-2 cell entry is mediated by the spike glycoprotein. The spike glycoprotein is composed of two subunits, S1 and S2, which mediate viral-host attachment and viral-host membrane fusion cascade, respectively [75]. SARS-CoV-2 spike recognizes and binds to the human ACE2 (hACE2) receptor through its receptor-binding domain (RBD) and is primed and activated by proteolytic cleavage by enzymes such as Furin and transmembrane serine protease 2 (TMPRSS2) [76, 77]. Spike, ACE2, Furin and TMPRSS2 have been shown to play a key role in mediating viral-host fusion attachment and fusion, and the Furin cleavage site on the Spike protein has been indicated as one of major reasons SARS-CoV-2 is so infectious [78, 79]. This makes these potentially promising drug targets for COVID-19 treatment [80, 81].

3.1.1 Drug repurposing targeting viral fusion

Inhibition of the Spike-Ace2 interaction is a primary target for drug repurposing as it is crucial to viral entry. *In silico* approaches have been performed and identified Simeprevir, an HCV NSP3A/4 protease inhibitor, as a potential blocker of Spike-Ace2 interaction by binding the RBD [82]. However, other *in vitro* studies showed that Simeprevir is not necessarily active against the RBD but is targeting the viral replication [83].

Another promising target is TMPRSS2, a serine protease. TMPRSS2 is associated to the host endothelial cell surface and cleaves the viral spike glycoprotein after binding to ACE2, activating it. The activation of spike protein then facilitates viral entry [84]. Camostat mesilate (a serine protease inhibitor) has been approved for the treatment of chronic pancreatitis, postoperative reflux esophagitis and kidney or liver disease fibrosis [81, 84, 85]. Since it is an established serine protease inhibitor it is a prime candidate to inhibit the TMPRSS2. An *in vitro* study indeed showed that camostat mesilate can suppress viral replication by halting the fusion of virus-cell membranes through the inhibition of TMPRSS2 [84]. Clinical trials using this drug are currently ongoing [86]. However, recently the results of a small double-blind randomized clinical trial using camostat mesilate performed in patients hospitalized with COVID-19. The trial determined no adverse effects of treatment with camostat but also no increase in positive clinical outcomes [87].

3.1.2 Drug repurposing targeting endocytosis of SARS-CoV-2

Besides direct membrane fusion, SARS-CoV-2 can also invade cells via endocytosis [88, 89]. This route involves several proteins that play an important role in endosome formation, such as two-pore channel 2 (TPC2), Cathepsin L (CTSL) and Vacuolar-type ATPase (V-ATPase) [90]. These proteins are indicated to be potentially interesting therapeutic targets for COVID-19 treatment.

Tetrandrine is a bisbenzylisoquinoline and calcium channel blocker, known for its anti-inflammatory, immunosuppressive, oncological, and cardiovascular bioactivity [48–49]. The compound has been shown to be effective in the treatment of silicosis [90–92]. According to an *in vitro* study, tetrandrine is a low micromolar inhibitor of viral replication that functions by blocking the two-pore channel 2 (TPC2), which impedes Ca^{2+} release which in turn prevents acidification of the endosome [92].

The now infamous anti-malarial drugs chloroquine (CQ) and hydroxychloroquine (HCQ) were also posited to inhibit endocytosis of SARS-CoV-2 [93, 94]. CQ and HCQ are potentially involved in blocking cleavage of spike by raising the pH of the endosomes, preventing cathepsin L-mediated proteolysis, which is a key element in membrane fusion after binding ACE2. Whilst many potential mechanisms of action have been suggested, none have been rigorously demonstrated. In addition, a recent large meta-data analysis has shown that there is no evidence that treatment with CQ or HCQ reduces COVID-19 mortality in patients [95]. To the contrary, evidence is available that shows HCQ is responsible for a small increase in mortality outcomes. These compounds garnered lots of attention when the presidents of prominent countries started promoting CQ and HCQ as wonder drugs that could combat COVID-19 [96]. However, as mentioned before most evidence points towards the contrary and the WHO recommends against the treatment with these drugs [97]. These cases have shown an important risk in the use of drug repurposing: in the age of hyper connectivity and social media echo chambers dangerous, unfounded ideas can avoid scrutiny and rigorous investigation, leading to large groups of people self-medicating with alternative treatments that have no scientific basis. This poses a problem in general drug development but even more so in drug repurposing cases where these compounds tend to be far more easily obtainable by the general public as they are approved and often available for purchase in pharmacies.

3.1.3 Drug repurposing targeting viral replication

After invading host cells, the coronavirus comes into the next stage of its life cycle: translation, replication, transcription and Assembly. This process mainly involves five different proteins: Mpro, RdRp, nsp14, MTHFD1 and Plpro, which have different functions [74, 98]. Mpro is also known as 3C-likeprotease (3CLpro) and proteolytically processes the majority of the polyprotein into functional polypeptides [99]. Similar to 3CLpro, Plpro is a viral protease that is responsible for cleaving polyproteins to generate a function replicase complex [100]. RNA synthesis, critical for viral replication, is performed by RdRP and its cofactors nsp7 and nsp8. Nsp14 has an exonuclease activity that supports RNA synthesis with an unusual RNA proofreading function. A study performed by Tinghua University showed that knockdown of MTHFD1, a key enzyme in cellular production of purine, dTMP and methyl groups, significantly inhibits viral replication [101]. As a result of their key functions in the viral life cycle these proteins are promising potential targets for antiviral drugs development. Clofazimine is an anti multibacillary leprosy drug which was approved for medical use in 1986. A recent study performed by The University of Hong Kong showed that clofazimine inhibits both viral spike glycoprotein mediated cell fusion and replication of SARS-CoV-2 *in vitro* [102].

Genome analysis has demonstrated that SARS-CoV-2 and SARS-CoV genes globally share >80% nucleotide identity and >89% similarity [73, 103, 104]. As a result, the key steps in the CoV family viral life cycle within the host cell are likely to be highly conserved. A key feature of this process is the expression of non-structural proteins (nsps). Subsequent to cell entry, two extended polypeptides (pp1a and pp1ab) from the CoV viral genome are generated by the host cell translation machinery [105, 106]. These two polypeptides then self-cleave into 37 distinct non-structural (nsp) proteins [107] and anaylsis has demonstrated that the CoV family possesses several proteases involved in this essential self-cleavage process: the papain-like protease (PLpro), and the 3C-like proteinases (3CLpro or Mpro) [108]. CoV generally encode two PLpros within nsp3, with the exception of gamma-CoV,

SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV-2 [100]. Thus, 3d unlike structural/accessory protein-encoding genes, which can show significant sequence variation in order to select between different potential host cell receptors, CLpro plays a central and critical role in CoV replication in host cells and the similarity in essential function leads to a high sequence similarity between the proteases of the CoV family, in particular beta-CoV [105, 106]. This increased similarity in the structure of the 3CLpro has the consequence of high structural between 3CLpro of different CoV family members—and the concomitant increased likelihood of cross-species function of CoV 3CLpro inhibitors and the potential to repurpose these inhibitors. The high sequence homology within Cov 3CLpros also provided high quality model templates—subsequently supported by the availability of high-resolution diffracting crystals—to perform both computational docking experiments, as well as molecular validation by X-ray crystallography [109]. There also exists the potential for the discovery and the development of a pan-anti-CoV inhibitor [110, 111].

This potential has been partially realized in not only the discovery of entirely novel SARS-CoV2 3CLpro inhibitors [99, 112, 113] (refs), but also in a number of reports describing successful identification of potential repurposing candidates [109].

For example, we and others have previously reported the results of a molecular docking experiment that indicated a class of well tolerated compounds (gliptins) as potential SARS-CoV2 inhibitors. For example, Anagliptin, a DPP4 inhibitor, is a well-established treatment for diabetes that is used by millions of patients. It has an excellent safety profile [114]. Computational docking demonstrated an efficient binding, with predicted H-bonds made to the backbone atoms of Gly163, Gly271, and Tyr268 and the side chain of Tyr273. Docking experiments also proposed that α-ketoamide inhibitors of hepatitis C virus (HCV) protease would be potential inhibitors of SARS-CoV-2 3CLpro. Efforts were concentrated on boceprevir and telaprevir as the docking poses were supported by experimental structure analyses (**Figure 1**). Subsequent biochemical assays demonstrated that boceprivir indeed displays strong binding to isolated 3CLpro of SARS-CoV2 and inhibits viral replication in cellular assays.

Similarly to CQ and HCQ, ivermectin, originally an anthelmintic, also gained widespread attention as a potential treatment for COVID-19 and some *in vitro* evidence of SARS-CoV-2 replication inhibition in cell cultures has been provided [115]. The suggested mechanism of action is the inhibition of importin alpha/beta-1 nuclear transport proteins which the virus uses to enter the nucleus and is an important part of the replication cycle as it suppresses host-immune response [116]. Despite the in vitro effect no clinical data has supported the therapeutic use of ivermectin at concentrations approved for use in humans. However, it gained attention in the media and people started buying ivermectin meant for use in large animals to self-medicate against the virus [117]. Even though ivermectin is approved for human use and is generally safe, the doses used in the treatment of animals are several times larger than recommended for humans and can cause side-effects ranging from mild diarrhea to seizures and coma. This once again shows the care that needs to be taken when repurposing drugs in how scientific results are communicated to the general public, as it can lead to potential dangerous situations [118].

3.1.4 Drug repurposing targeting immune response modulators to treat SARS-CoV-2

Clinical symptoms resulting from SARS-CoV-2 infection are heterogeneous. Recent reports have shown that the cytokine storm effect may play a significant

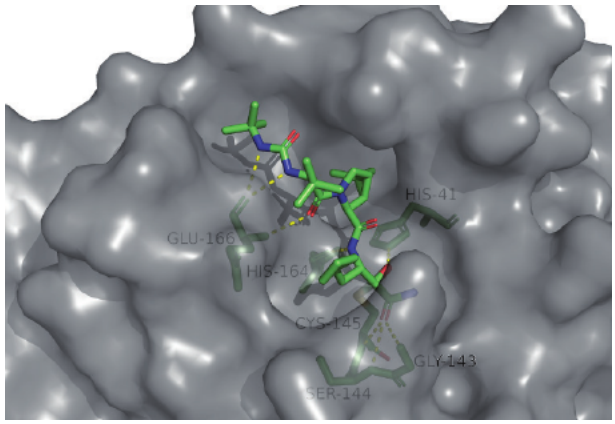

Figure 1.
Boceprevir (green), a HCV NS3/4A protease inhibitor, bound to SARS-CoV-2 3C-like protease (gray surface representation) with several key drug-protein interactions shown. Hydrogen bonds are shown as yellow dotted lines. PDB accession code: 6zru.

role in disease progression, potentially leading to multiple organ failure and death [119]. Immune response-related proteins have been proposed as potential targets for treatment options [120, 121]. Even though effectively suppressing cytokine storm does not directly combat the viral infection itself, it can be a crucial treatment option against COVID-19. It has previously been demonstrated that melatonin has beneficial effects on infection induced models of respiratory disease and associated complications [122]. Recently evidence also surfaced that it can inhibit COVID-19 induced cytokine storm [123]. Tocilizumab, an interleukin antagonist used for rheumatoid arthritis, is an immunosuppressor and was thus posited to be effective at reducing inflammation caused by SARS-CoV-2 [124]. It has recently become one of the first drugs to be recommended by the WHO as an effective treatment against COVID-19 [125]. Even though this is very promising there is an issue with the availability and affordability of this drug [126].

Researchers at Johns Hopkins found that the drug prazosin, an alpha-1 blocker used to treat high blood pressure, can prevent cytokine storms and that it significantly strengthened survival following inflammatory stimuli in preclinical models. This study is now in clinical trials [127].

3.1.5 Drug repurposing targeting pyroptosis

Sepsis is a systemic inflammatory response syndrome reulsting from dysregulation of host immunity. It is one of the deadliest clinical symptoms of severe SARS-CoV-2-infected patients [128]. Sepsis treatment normally mainly relies on the administration of intravenous antibiotics. However, since SARS-CoV-2 viral sepsis is not bacterial in nature the efficacy is low. Treatment is difficult, typically consisting of supplying oxygen and assisted breathing using a ventilator. Cocktails of antivirals and immune suppressors are also given but usually only have limited effect [129]. Approximately one-third of the discharged patients will die and one-sixth will suffer severe persistent impairments in the following year [130]. These facts taken together demonstrate the urgency and significance to find new treatments.

Sepsis is associated with pyroptosis (inflammatory programmed cell death) that is triggered by proinflammatory signals [131]. When viruses invade the host cell, inflammasomes are activated which in turn triggers an inflammatory response [132]. Pore-forming protein gasdermin D (GSDMD) is cleaved by activated Caspase-1, releasing its N-terminal domain [133]. The GSDMD N-terminal domain

induces the formation of a large plasma membrane pore, resulting in pyroptosis [134]. Under normal circumstances pyropthosis can be good response, being able to trigger cell death of infected cells, releasing the pathogens and stimulating subsequent phagocytosis, protecting against infections [135]. However, excessive activation of pyroptosis will exacerbate sepsis or excessive cell death, causing immunity dysregulation [136, 137].

Disulfiram is approved for the treatment of chronic alcoholism. In a study conducted by Boston Children's Hospital, researchers found that disulfiram posesses inhibiting potential towards GSDMD both in *in vitro* cell assays and in *in vivo* mouse experiments. The experiment results showed that disulfiram inhibit the formation of the GSDMD pore by covalently modifying Cys191 of human GSDMD [138]. These results indicate that disulfiram could potentially be used to combat the pyroptotic effects induced by sars-cov-2 infection, hopefully reducing the negative clinical outcomes.

3.2 Drug repurposing for other viral diseases

Not only large global pandemic diseases are worth investigating for drug repurposing opportunities. Smaller, localized viral epidemics still plague many countries to this date. These diseases tend to fly under the radar since they typically occur in poorer regions of the world, meaning less research money is being spent on novel drug development. Drug repurposing could be the solution for these diseases due to the far faster and cheaper development pipeline.

3.2.1 Dengue virus

Dengue virus (DENV) is a single-stranded RNA virus, enveloped by a bilayer lipid membrane. The premembrane (prM) protein and envelope glycoprotein adhere to the membrane. Dengue virus can infect humans through mosquito bites. Symptoms, that include high fever, severe headache, muscle and joint pain, nausea, vomiting, swollen lymph nodes and rash, usually appear 3–14 days post-infection [139]. Most patients will recover in 2–7 days, while a small number of patients' conditions may worsen accompanied by bleeding, thrombocytopenia and plasma protein effusion. Up to 22,000 people die from Dengue annually and currently there are no therapies to treat this infection [140].

Ulipristal, a FDA approved small molecule, is an elective progesterone receptor modulator (SPRM), that has been demonstrated to be a potent inhibitor of DENV, most likely by blocking viral entry [141]. The antiviral activity was evaluated by *in vitro* DENV infection assay using Vero E6 cells. The results show that ulipristal has an antiviral effect against DENV in Vero E6 cells with an EC50 of 8.3 ± 0.1 μM. The anti-DENV effect of ulipristal was further confirmed using a murine infection model. The ulipristal-treated group presented less weight loss and disease symptoms compared the control group. A significant drop was also detected in the degree of viremia in the blood of the ulipristal-treated group. This study showed that ulipristal has desirable anti-DENV effects *in vitro* and *in viv*o [141].

3.2.2 Zika virus

Zika virus (ZIKV) is another virus that is propagated by mosquitoes and belongs to the genus of flaviviruses. ZIKV infection generally causes only mild symptoms, including fever, rash, conjunctivitis, muscle and joint pain, and headache. However, it has shown severe tetatogenic impacts, being able to cause a range of neurological complications, such as Guillain-Barre syndrome and microcephaly, in the fetuses of infected pregnant women [142].

There are no currently approved specific therapies for ZIKV infection [143]. However, a screening study utilizing 774 approved drugs has shown promising results. In vitro studies showed that ivermectin (anthelmintic), mycophenolic acid (an immunosuppressant), and daptomycin (a lipopeptide antibiotic) can inhibit ZIKV, resulting in reduced infection rates [144].

3.2.3 Ebola virus

Ebola virus is one of numerous hemorrhagic fever viruses, which was first discovered in 1976. It can cause severe viral haemorrhagic fever with case fatality rates vary from 25 to 90% [145]. It is characterized as a non-specific febrile illness (symptoms may include anorexia, arthralgia, headache, malaise, myalgia and rash) in the early infection and progresses to severe gastrointestinal symptoms (nausea, vomiting and high-volume diarrhea) in the first week [146]. To date, a monoclonal antibody (mAb114) and a cocktail of three antibodies (REGN-EB3) have been approved for the treatment of Ebola [147, 148]. Besides these biologics there has also been attempts at drug repurposing for this disease. Several drugs such as Amiodarone (anti-arrhythmia), bepridil (anti-angina pectoris), teicoplanin (antibiotic), amiodarone (ventricular fibrillation/tachycardia) and favipiravir (RNA polymerase inhibitor) have shown therapeutic potential for Ebola, but their efficacy requires further confirmation [149–151].

3.2.4 MERS-CoV

A warning of the potential for a coronavirus pandemic was provided by the Middle East respiratory syndrome coronavirus (MERS-CoV). While the impact of this outbreak was significantly less than that of the current SARS-CoV2 outbreak the urgent need for MERS-CoV treatments was recognized, also including a focus on repurposing approaches and a call for the development of pan-corona virus inhibitors [152]. Suggested repurposing agents included GS-5734, which has previously demonstrated antiviral against multiple viral families, including *Coronaviridae*. GS-5734 activity *in vitro* was supported by reduced disease effects in mouse models and, while resistance mechanisms emerged, they were associated with a loss in viral fitness *in vitro* and *in vivo*—supporting the further analysis of GS-5734 as a pan-corona inhibitor [153, 154].

Similarly, lopinavir-ritonavir (a molecule designed as an inhibitor of the HIV-1 protease inhibitor) was proposed as a repurposing target of the 3CLpro of both SARS-CoV and MERS-CoV during their respective outbreaks [155, 156]. Combination therapy approaches in both cases resulted in improved patient outcomes, thereby offsetting the lacking of designed affinity that is a hallmark of repurposed compounds. In the example of SARS-CoV, a study on a combined therapy with ribarivin (a guanosine analog with activity against multiple viral families that inhibits viral RNA synthesis by RdRp) demonstrated both reduced viral load and improved clinical outcomes [157]. Whereas, a clinical trial of lopinavir-rotonavir in combination with IFN-β1b targeted therapies was proposed for MERS-CoV patients in Saudi Arabia [158]. Ribarivin itself was also a focus for repurposing during the SARS-CoV and MERS-CoV outbreaks. However, while efficacy of ribarivin alone could be demonstrated *in vitro* the doses required for a clinical response could not be supported by patients [159, 160].

Screening of an FDA-approved compound subset against viral replication in culture identified lopinavir and an additional 3 compounds with IC50 values in the low micromolar range (chloroquine, chlorpromazine, and loperamide) [156]. This again demonstrates not only the potential for experimentally based repurposing screens

to identify potential agents, but also suggests that the relatively limited potency of the agents identified may require the assessment of combination therapies to provoke a clinical response. This additional limitation of identifying appropriate combination therapies may well represent a common theme as a complicating factor in repurposing strategies.

4. Conclusions

In summary, the relatively conserved elements of the viral life cycle offer many opportunities to reexamine compounds developed to address previous outbreaks for efficacy against novel outbreaks. Clear examples are shown in the results against non-structural proteins above, which often maintain significantly higher sequence homology across species due to a conserved mechanism than structural proteins. However, while this sequence conservation indeed leads to a degree of "cross-talk" between nsp inhibitors, the required exquisite and intricate nature of the interaction between a successful drug and its target will almost inevitably reduce the efficacy of a monotherapy. As a result, it is likely that while repurposing can identify promising candidates, care must be taken not to hope for a single effective solution in existing drugs (e.g. Ivermectin, hydroxychloroquinine, etc.). Rather, functional (clinical) solutions are much more likely be found in careful clinical trials of combination therapies of drugs identified through repurposing screens.

The current combination of virtual, *in vitro* and *in vivo* screening is well positioned to perform rapid repurposing experiments on the relatively small number of clinically approved candidate molecules. However, significant research effort should be expended globally to continue to identify potential viral inhibitors and further populate the potential repurposing list.

Response speed is a key factor facing outbreaks. Drug repurposing is a practical solution that provides multiple benefits beyond classical drug discovery. Perhaps the greatest advancement in this area has been the improvements in computational techniques, that has developed in parallel with advances in structural biology—both of which continue to improve. These structural views of the proteins driving disease expand the number of experiments that can be performed *in silico*, providing both an increase in speed of hypothesis generation, as well as an important pre-filter stage to select out candidate molecules for screening. In our opinion a key aspect that should not be overlooked is the *in vitro* validation of a molecular effect on a proposed target. Certain recent experiences have shown that attempts to bypass this stage and short-cut the process by directly jumping into clinical trials can produce conflicting results, leading to confusion and loss of confidence of the public. However, despite successful application of drug repurposing, no single golden standard as yet exists to give relatively predictable results.

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Conflict of interest

The authors declare no conflicts of interest in the contents of this manuscript.

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Antituberculosis Drug Repurposing: A New Hope for Tackling Multi-Challenging TB in Timely Manner

Shahnawaz Majeed, Safiya Mehraj and Zahoor Ahmad

Abstract

Tuberculosis still stands as the world's leading infectious disease as 1/4th of the world's population harbors Latent TB infection (LTBI) > 10 million develops active TB and ~ 1.5 million people die per year. Approximately 4,65,000 people fell ill with multidrug or rifampicin-resistant tuberculosis (MDR/RR-TB)/year. This deadly TB scenario demands new TB drug regimens to tackle global infection reservoir, and worldwide spread of drug resistance and DS TB. Successful entry of single new drug into market is much complicated mission owing to time, cost, efficacy, and safety issues. Therefore, drug repurposing seems one reliable hope to meet the challenges of modern TB drug discovery timely, as it starts with examining market acclaimed drugs against other diseases for their efficacies against tuberculosis avoiding several lengthy and costly steps required for new molecules. Several drugs have been identified, which show potential for TB treatment. There is need for careful consideration of various trial designs to ensure that TB phase III trials are initiated for fruitful development of new TB treatment regimens. TB drug repurposing will not only give fast track novel drugs but will also serve to identify new targets for future development in cost-effective manner.

Keywords: extensively drug-resistant TB, drug repurposing, clinical trials, computational strategies, antibacterial, antifungal, antiprotozoal, immunomodulators

1. Introduction

Drug repurposing, synonymically, known as drug reprofiling, drug repositioning, drug re-tasking, drug redirection, drug recycling, drug rescuing, and therapeutic switching, is a strategy of identifying new pharmacological applications for an approved or investigational drug that are beyond the original scope of its medical indication. It can also be defined as use of the new drugs for the additional diseases other than its already intended use. It establishes new therapeutic uses for already known drugs, which are approved, abandoned, discontinued, or experimental drugs [1, 2]. Need for drug repurposing surfaced due to multifold challenges faced by global pharmaceutical industry [3]. Bringing new drugs into the market with changing regulatory requirements costs huge economy and time. Return benefits are lesser than the expenditure needs on research and development (R&D) [4], and this demoralized the investors from investing in pharmaceutical industry. Repurposing

a drug, on other hand, has lesser possibility of failure from a safety point of view because the repurposed drug has already been found to be adequately safe in pre-clinical models provided early-stage trials have been completed. Secondly, the time duration for development of drug can be reduced, as most of the safety assessment, preclinical testing, and, in some cases, formulation development are already completed. Thirdly, less expenditure is needed, though varies with the stage and process of development of the repurposing candidate. On average, on traditional drug discovery takes 5–7 years, and failure rate of 45% associated with only toxicity issues keeps the effort and cost of almost one decade at stake [5, 6]. Repurposed drug, in contrast, saves time and effort for preclinical, and phase I and II trials, although phase III and regulatory costs may remain more or less the same (**Figure 1**).

It is estimated that it takes on average 13.5 years to bring a new molecular entity to market, Drug repurposing is based on previous research & development, allowing compounds to progress through the drug development process more quickly as well as saving on the substantial costs associated with previous attrition [7]. It is well known that *de novo* drug discovery and development is a 10–17-year process from idea to marketed drug [8]. The probability of success is lower than 10% [9]. Drug repositioning offers the likelihood of abridged time *and* risk as several phases common to *de novo* drug discovery and development can be bypassed because repositioning candidates have frequently been through several phases of development for their original indication. ADMET, absorption, distribution, metabolism, excretion and toxicity; EMEA, European Medicines Agency; FDA, Food and Drug Administration; IP, intellectual property; MHLW, Ministry of Health Labour and Welfare.

Repurposing cost of a drug from lab to market is estimated to be US$300 million on average, compared with an estimated ~$2–3 billion for a new chemical entity [10]. The cost of developing a new drug has soared to $2.6 billion [11], which has given drug repurposing strategy a substantial momentum to cover one-third of the total approvals given for new drugs and generate around 25% of the annual revenue for the pharmaceutical industry (**Figure 2**) [12].

Moreover, 30% of the US Food and Drug Administration (FDA) approved drugs and biologics (vaccines) constitute repurposed candidates. The global market for

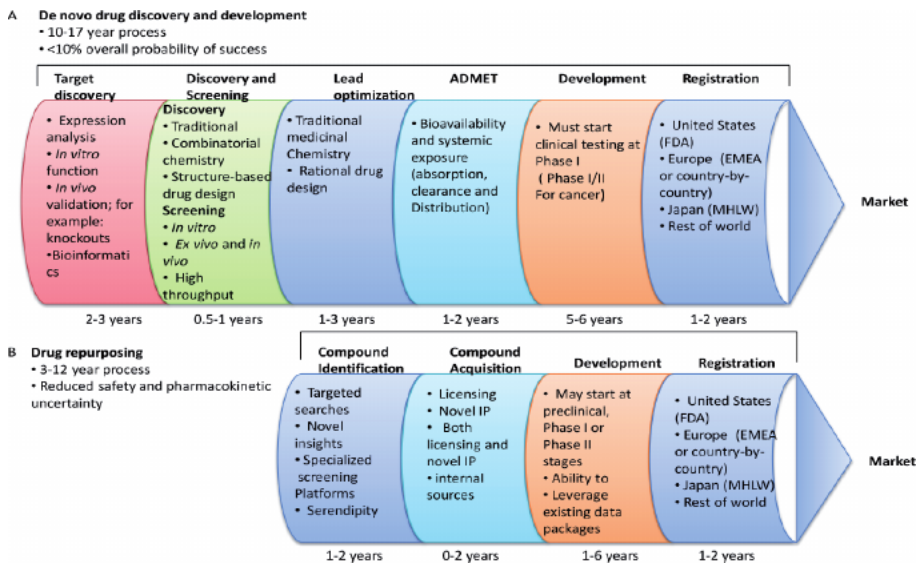

Figure 1.
Traditional drug discovery versus drug repurposing/A comparison of traditional de novo *drug discovery and development versus drug repositioning.*

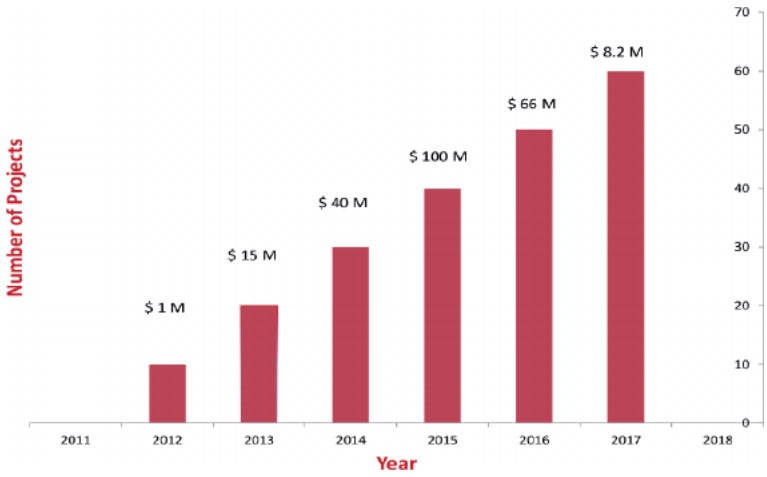

Figure 2.
Bar chart representing year-on-year trends on funds granted for drug repurposing projects in the recent past (2012–2018), with the advent of time the drug repurposing projects are increasing with the increase of expenditure of funds granted for various repurposing projects. Funds raised for drug repurposing projects increased consistently from 2012 (US$1 million) to 2015 (US$100 million). In 2016, although the funds raised were comparatively low, there were more drug-repurposing projects initiated (47 projects). This emphasizes the fact that drug repurposing has gained traction in the recent past [13].

drug repurposing is valued at 18 million US$ in 2018 is expected to reach 35 million US$ by the end of 2025, growing at a CAGR of 30% during 2019–2025.

2. Scope of drug repurposing

Biggest interest of drug repurposing is the reduction of time and cost for achieving new drugs for disease. It also can be a source of treatment options for lesser known and rare diseases. Novel methods based on databases have been proposed to tackle diseases by repurposing of drugs. A suitable data organization can provide a web tool to facilitate the repurposing drugs to treat old and new; common; or rare diseases. But still use of such data is not widespread, though the benefits are well established and calculated. However, drug repurposing might turn out to be expensive, time consuming, and risky. Moreover, certain legal bumps make the road to drug repurposing tougher. Despite all these limitations, drug repurposing still promises of great scope if given better incentives, structured guidelines, and support. Currently, statistical screening of the approved drug can help find repurposing goal of the drug *via in silico* techniques to screen wide library of compounds and target data for successful repurposing technology. But influence of target is not much explored as is expected. The original and repurposed target exploration can yield information about similarities and dissimilarities, which can help to know about binding affinity of the drug. This aspect further needs molecular level study to strength the drug repurposing process. Globally, there are numerous diseases without suitable therapeutic options. Rapidly advancing understanding of human biology, increasing pool of actively studied moieties, and the need to produce cost-effective therapies are driving the need to study the existing set of molecules for relevance across multiple diseases. The promise of cost effectively realizing the full potential of existing drugs *vis à vis* new therapeutic purposes is too attractive for all stakeholders in the healthcare value chain—patients, providers, pharma, and payers, to pass (**Figure 3**).

Drug repurposing began serendipitously; however, with increasing interest from pharmaceutical companies and the identification of various bioinformatics

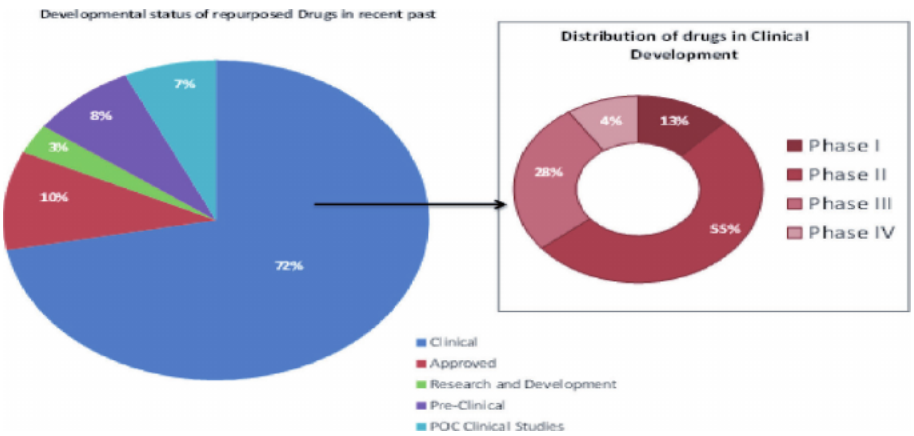

Figure 3.
The developmental status of repurposed drugs from 2012 to 2018.

and cheminformatics methodologies, it has evolved into an innovative, data-driven, cutting edge strategy. To understand the recent impact of drug repurposing on drug discovery and development, data on repurposed drugs were collated from Excelra's proprietary drug repurposing portal, news bodies, and social networking sites, and then analyzed to reveal any drug repurposing trends. From 2012 to 2017, almost 170 repurposed drugs entered the drug development pipeline. Currently, these drugs are at different stages of development. Most (72%) are in clinical development, especially Phase II, 7% are in PoC clinical studies, 8% in preclinical stages, 3% in research and development, and 10% have been approved [13].

2.1 Challenges in drug repurposing

Despite being an attractive drug option with multiple benefits, drug repurposing is a complex technology met with many challenges. The biggest challenge is to choose the approach to make full use of massive amounts of medical data. The issue of limited intellectual property (IP) protection for repurposed drugs is another challenge as IP protection to repurposed drugs is much limited [14]. On the other hand, IP protection of the old drugs prevents them from entering market as repositioned drugs. Moreover, forced closure of some repositioning projects happens due to risk for wastage of time and money [15]. An important principle in drug repurposing process is market exclusivity, which is defined as "method of use" patents valid for a period of 20 years. Conventional drug development process is characterized by "composition of matter" patents while the repurposing process is considered more contestable. "Composition of matter" is protected by the strongest patent protection [16] and is more easily attainable from *de novo* drug development, while as "Method of use" patents that cover repurposed drug can be challenged as merely incremental advances. However, under the right circumstances, a "method of use" patent can be as effective as a "composition of matter" patent in protecting a repositioned drug product depending on the availability of generic products to be substituted through off-label use to achieve expected results with the repurposed drug. The FDA allows physicians off-label prescription of drugs, but prohibits offline marketing of drugs by pharmaceutical companies [17]. IP issues act as barrier for marketing of certain repurposed drugs [18]. There are bleak chances for physicians to prescribe drugs without clinical trial evidence to support the new use; however, "composition of matter" protection may be available for repurposed drugs. Hence, from a legal perspective, a careful consideration of intellectual property rights and

acts is imperative. For antimicrobial reuse of agents, more limitations are added to the already described list. A big limitation of dosage, toxicity, and resistance development for re-purposed drug is a challenge in itself [19, 20]. If non-antibiotic drug is repurposed for infectious disease, efficacy is usually achieved at much higher doses than of those specified in the original registration, toxicity, and adverse events raise a concern. Another limitation is pharmacokinetic profiles of drugs, which upon repurposing might not serve the benefits which it served for the original use. This limitation affects antibacterial use of drugs as, plasma protein binding plays a major role and also impairs antimicrobial activity as it might narrow the therapeutic index for the antimicrobial indication. Thus, suitable pharmacokinetic profile is a big challenge effecting credibility of drug candidate for repurposing. A major limitation in the drug repurposing is the expenditure needed for clinical trials. Pharmaceutical companies show lesser interest in investment for clinical trials of repurposed drugs as these are usually generics or start with expiry of patent lifetime, there is little scope of turn over for companies. Solutions to address this problem have included raising economic support from public sources, as such sources prioritize health outcomes over commercial motives. Smaller clinical trial set can also be a set for repurposed drugs and such trials are designated as Phase II trials. But clinicians do not consider them much valid, even if high-quality data are generated. However, drug repurposing can be a practical approach, but the issues of funding and feeble interest of pharmaceutical industry hamper the prospects of its clinical usage.

3. Tuberculosis (TB)

TB continues to be threat to public health enlisted among top 10 causes of death worldwide. The causative agent, bacillus *M.tb,* singly kills more people than HIV/AIDS pathogen does. It is one of the momentous disquietude since two decades when the World Health Organization declared it a global health emergency. With the rise of antibiotic resistance in *M.tb*, the causative agent of TB has made it immensely difficult to control the disease with the already existing anti-TB chemotherapy. The need of hour is to develop effective drugs with novel mechanism(s) of action so as to curb the drug resistance. The development of novel chemical entities requires >10 years of research, with high-risk investment to become available commercially. TB spreads are easier as it is contracted by inhaling droplets of infection expelled in air from TB patient. TB mostly affects lungs (pulmonary TB) but can also affect all other sites (extrapulmonary TB) sparing only nail and hair. About a quarter of the world's population is infected with *M. TB*. TB continues to be a major cause of morbidity and mortality, primarily in low-income and middle-income countries [21]. In 2019, an estimated 10.0 million (range, 8.9–11.0 million) people fell ill with TB—in HIV-negative people, 1.2 million (range; 1.1–1.3 million) TB deaths, and 208,000 deaths (range; 177,000–242,000) among HIV-positive people. Men (aged ≥15 years) accounted for 56% of the people who developed TB in 2019; women accounted for 32%; and children (aged <15 years) for 12%. Among all those affected, 8.2% were people living with HIV (World Tb report 2020). Eight countries accounted for two-thirds of the global total: India is a leading country, which covers (26%) of TB burden, Indonesia (8.5%), China (8.4%), the Philippines (6.0%), Pakistan (5.7%), Nigeria (4.4%), Bangladesh (3.6%), and South Africa (3.6%). In 2020, we have lost count of TB-affected people and deaths due to COVID-19 pandemic and the previous efforts against TB as well. More DOTS centers got malfunctional due to medical emergency, and many children missed the BCG vaccination. Till emergence of drug-resistant strains, TB was successfully treated using

chemotherapy which comprised of four first-line anti-TB drugs: isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB). Then, second-line drug regimens were developed, which consisted of aminoglycosides (Kanamycin, amikacin), capreomycin, cycloserin, para-aminosalicylic acid, thioamides (ethionamide (ETH), prothionamide), and fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin) [22]. But multidrug-resistant, extremely drug-resistant, and total drug-resistant strains emerged and the conventional drug regimens started to lose their efficacy. Incomplete, inadequate, and wrong prescription of the standard therapy are responsible for the emergence of drug-resistant strains of *M. TB* [23]. Multidrug-resistant TB (MDR-TB) is defined as resistance to at least isoniazid and rifampicin. Extensively drug-resistant TB (XDR-TB), which causes more severe disease manifestations, is not only resistant to isoniazid and rifampicin but also resistant to any fluoroquinolone and injectable second-line aminoglycosides. When the pathogen becomes resistant to all first- and second-line anti-TB drugs, totally drug-resistant (TDR) is said to have developed. TB existing drugs are slow to eradicate the pathogen in patients and the intrinsic resistance systems of *M.tb* have evolved to make the present antibiotics ineffective [24]. Moreover, long-term chemotherapy with frequent dosage arises chances of drug toxicity; therefore, urge for new drugs is on rise to shorten the TB treatment. The birth of drug repurposing in TB treatment was marked upon global resurgence of TB, especially in New York City during the late 1980s where the infection had almost quadrupled and more than one-half of cases were resistant to INH and RIF (i.e., MDR). Like cancers or other diseases, drug repurposing approach for TB is based on various approaches such as host-directed targets, structure-based drug targets, *in silico*-based approach, and combinatorial drug therapy approach. In this book chapter, we provide an overview of various approaches that aid drug repurposing for TB. We also discuss the targets and clinical trials carried out for the repurposing strategy.

3.1 Tb drug development

Mid-twentieth century is engraved as golden era in history of antibiotic discovery when streptomycin got discovered and discovery of major classes of antibiotics was initiated using actinomycetes [25, 26]. Decades later, use of semisynthetic compounds as antimicrobials was focused upon as the bugs developed resistance against previous antibiotics [27]. However, Bedaquiline (TMC207), the first FDA approved TB drug for 40 years, was discovered with *Mycobacterium smegmatis* as surrogate but many other good leads are supposed might have been missed in the past [28]. In modern times, the drug development strategy has been updated and two basic approaches are followed *via* phenotypic screening or empirical approach, which involves evaluating the molecule of interest by studying the phenotypic changes it induces in cells, tissues, or whole organisms [29]. The other approach is target-based screening wherein the molecule of interest is screened alongside a precise enzyme *in vitro* [30]. Result of phenotypic screening was small molecule-based drugs that were accepted and approved by FDA between 1999 and 2008 [31]. Consequently, various companies (e.g., Novartis AG and GlaxoSmithKline) and research centers pay attention on phenotypic screening as a considerable device for the process of drug discovery [32]. This approach has been used to screen the inhibition of cell growth [31] and turn out to be successful with clinical-stage anti-TB drugs, such as nitroimidazoles (delamanid and pretomanid) [33], 1,2-diamine SQ-109 [34], and bedaquiline [28]. Two years before, in 2019, this drug discovering approach was approved by FDA for pretomanid for the cure of adults with pulmonary multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) that were non-responsive or treatment-intolerant (www.tballiance.org). Combination of drug Pretomanid along with three-drug linezolid and bedaquiline was approved for 6

months, in oral dosage (cooperatively referred to as the BPaL regimen) [35]. But there are certain drawbacks associated with this approach. For example, the high expenditure and ambiguity that is related to phenotypic screening in the process of drug discovery limits its progress in drug development [36]. Target-based approaches are based on the finding effect of drug on certain specific target and are more focused in the preclinical phase of drug development toward the chemical lead optimization and toxicology studies. In 1998, with sequencing of whole *M.tb* genome revealing 4.4 million base pairs and 4000 genes, knowledge about potential *M.tb* targets got broadened and target-based approach was expected to yield successful results [37]. Target-based screenings have not yielded promising outcomes due to a few reasons such as: (i) permeability of purified enzyme or target to enter screens; (ii) non-specific nature of the molecule to inhibit the target; and (iii) compounds are not constantly effectively bioavailable orally [38]. Likewise, obtaining compounds with cell permeability and without cytotoxicity through medicinal chemistry may be a very time-consuming and intricate process [39]. Target-based approaches need evaluation of target features that include target essentiality, vulnerability, and novelty. A potential target ought to be essential part of fundamental survival or virulence of the pathogen both in active and in latency modes. It shall not be part of human host to avoid toxicity. To evaluate the targets, the essentiality of a target gene is established by mutant generation [40]. To recognize hypothetical proteins as druggable targets in XDR-TB strains, computational subtractive genomics approach has recently been employed [41]. As pointed out by [37], inhibition of ATP Synthase in particular and the energy metabolism are highly druggable targets as confirmed by these findings. To conclude, it is enviable that a three-dimensional structure of a protein target be accessible to help guide medicinal chemistry efforts [42]. By explanation, drug discovery implies exploration of unknown. Though the process of drug discovery might be predisposed by target, all knowledge about the target, a phenotypic product, or precise profile of chemical compound must be screened while selecting molecule for the first time. These, in turn, correspond to biases, which might exert influence on the outcome of choices that are measured as successes, as The "rule of five" put forth by *Lipinski* [43] that is based on the physicochemical profiles of drugs in phase II and the set of rules put forth by Veber [44]. In the process of lead optimization and/or in the process of drug development, improved oral bioavailability in rats serves as guiding principle. ADMET characteristics are enhanced when CLogP <4 and MW <400 Da as recommended by Gleeson [45]. Antimycobacterial drugs/agents do confront rules that are already reputable because they are more lipophilic as recently being reported [46]. The overall drug development process based on phenotypic screening or target specific seems very cumbersome process, and still, successful drug regimen is yet to be achieved. Therefore, approaches of drug repurposing for Tb are essential to be focused upon.

3.2 Approaches of drug repurposing in TB

Host-directed approaches/therapy: host-directed therapy (HDT) is used to target pathogen-exploited pathways in the host. This therapy makes use of repurposed drugs, antibodies, vitamins, small molecules, as adjuvants to support the conventional treatment. Pulmonary diseases, involving uncontrolled healing mediated by profibrotic cytokines, are considered as autoimmune diseases. Such pulmonary pathologies usually do not respond to the standard anti-inflammatory agents. TB also represents this kind of pathophysiology. Interferon-γ, an adjunct, is delivered subcutaneously for chronic granulomatous disease and osteopetrosis. Interferon-γ stimulates macrophage function and inhibits fibrotic pathways. Interferon-γ has been repurposed as an inhaled aerosol, targeting directly to the lung so, to treat many diseases exaggerated by dysregulated immunity like TB. Inhalation of

interferon-γ has been studied as potent antitubercular adjuvant in a clinical trial against MDR-TB by Condos *et al* and has been found effective [47]. Elevated levels of *IRF-1*, *IRF-9*, and *STAT1,* from lung segments in BAL cells, were visualized when in other trial co-administration of anti-TB drugs and IFN-γ were given to TB patients. IFN-γ provided potential to be used as an adjuvant therapy as it energetically stimulated gene expression and signal transduction in alveolar macrophages of TB patients. In addition to a chemotherapeutic cocktail, IFN-γ has been evaluated as an adjuvant therapy *via* other approaches. An intramuscular injection of IFN-γ as an adjuvant chemotherapy for a time period of 6 months led to the reduction of lesion sizes, cultures, negative sputum smears, and increased body mass index [48].

3.2.1 Pathogen-directed approaches

Growth of heterogeneous *M.tb* populations during infection is an important factor for antibiotic tolerance. Inside phagolysomes, acidification alters the redox physiology of *M.tb*, which alters the bug to replicate into population of drug-tolerant strains. The mechanism behind this tolerance has been elucidated with RNA sequencing of redox-altered *M.tb* population; and involvement of iron-sulfur (Fe-S) cluster biogenesis, hydrogen sulfide (H2S) gas, and drug efflux pumps. Chloroquine (CQ), an antimalarial drug inhibited phagosomal acidification, improved lung pathology and reduced post-chemotherapeutic relapse in experimental animal models. The pharmacological parameters of CQ did not show any significant drug-drug interaction with first-line anti-TB drugs upon co-administration in mice. A link between phagosomal pH, redox metabolism, and drug tolerance in replicating *M.tb* is suggestive of repositioning potential of CQ against TB and a relapse-free cure [49]. One of the determinants of *M.tb* virulence is protein phosphorylation. Unique tyrosine-specific kinase, protein tyrosine kinase A (PtkA), present in the *M.tb* genome phosphorylates protein tyrosine phosphatase A (MptpA) and increases PtpA activity and pathogenicity. Several proteins including the cyclophilins are essential for biofilm generation. *M.tb* cyclophilin peptidyl-prolyl isomerase (PpiB), interaction cyclosporine-A, and acarbose (US FDA-approved drugs) were predicted by *in silico* docking studies. Further surface plasmon resonance (SPR) spectroscopy was used to confirm the inhibition in growth of *M.tb*. Gallium nanoparticle (GaNP) reported to have bactericidal effect, when used with Cyclosporine—additionally disrupted *M.tb* $H_{37}R_v$ biofilm formation. Co-culturing *M.tb* in their presence resulted in significant (2–4-fold) decrease in dosage of antitubercular drugs such as isoniazid and ethambutol [50]. Targeting MurB and MurE enzymes involved in the muramic acid synthesis pathway (Mur Pathway) in *M.tb* has been studied and FDA-approved drugs from two repositories, that is, Drug Bank (1932 drugs) and e-LEA3D (1852 drugs), have been screened against these proteins. Binding-free energy and hydrogen bonding interactions have been seen to effect the stability of interactions among drugs and drug sites. Sulfadoxine (−7.3 kcal/mol) and pyrimethamine (−7.8 kcal/mol) showed stable interaction with MurB. Lifitegrast (−10.5 kcal/mol) and sildenafil (−9.1 kcal/mol) showed most reliable interaction with MurE. Hence, these characteristics of drugs for repurposing are supposed to be further explored to achieve efficient repurposing of the drugs [51].

3.2.2 In silico *approach*

Several computational approaches have been developed to discover new repurposing opportunities and integration of these approaches can help rediscovering drugs with more chances of success as prediction of new drug-target interaction, target-disease, and drug-disease associations can be done more rationally. Based on systemic data analysis of host, pathogen, or drug which

include signature *matching* gene expression, chemical structure, genotype, or proteomic data or Electronic health records (EHRs) can help to formulate repurposing hypotheses for various drugs [52].

3.3 Signature matching

It is defined as the unique characteristics or "signature" of a drug which upon comparison with another drug, disease or clinical phenotype can yield another purpose of the drug [53]. Uniqueness of a signature owes to its chemical structure or changes transcriptomic, proteome, metabolome, or adverse event profiles that are generated upon its administration. Matching the signatures can be used to make drug-disease comparisons (estimating drug-disease similarity) [54] and drug-drug comparisons (drug-drug similarity) [55] and the correlation between the two defines the potential effect of drug on the disease [56]. Publicly accessible gene expression data of drugs and diseases have been mapped for easier drug repurposing predictions. Such an application is Connectivity Map (cMap), established in 2006 by the Broad Institute, and has been a success to predict drug-disease interactions. Other repositories such as Gene Expression Omnibus and Array Express that contain raw gene expression data from hundreds of disease conditions based on chemical structures with that of another drug to see whether there are chemical similarities could suggest shared biological activity. Upon selecting a set of chemical features for each drug a network is constructed based on the shared chemical features and is called the statistics-based cheminformatics. This approach was undertaken by Keiser and colleagues [2] to predict new targets for 878 FDA-approved small-molecule drugs and 2787 pharmaceutical compounds. Another such approach called similarity ensemble approach (SEA) evaluated the structural similarity of drug to target's ligand set, which led them to identify 23 new drug-target associations. But this approach has its limitations of errors in chemical structures and their physiological effects [54]. The signature-based approach has limitation of difficulty in mining adverse effect information from drug package inserts and the lack of well-defined adverse effect profiles and causality assessments for a number of drugs. However, artificial intelligence technologies that can undertake text mining and natural language processing represent potential future opportunities to overcome these limitations.

3.4 Molecular docking

It is a structure-based computational strategy to predict binding site complementarily between the drug and the target [57]. It might involve conventional way wherein in [19] multiple molecules are tested against that particular target which is been already identified. Conversely, drug libraries could be explored against an array of target receptors (inverse docking: several targets, and one ligand) to identify novel interactions that can be taken forward for repurposing. This approach was used by *Dakshanamurthy and colleagues* [58] on 3671 FDA-approved drugs across 2335 human protein crystal structures to repurpose mebendazole, an antiparasitic drug, to inhibit vascular endothelial growth factor receptor 2 (VEGFR2), a mediator of angiogenesis. This approach has certain pitfalls, like unavailability of protein 3D structures [59].

4. Genome-wide association studies (GWAS)

This is a computational approach aimed to identify genetic variants associated with common diseases to unveil disease mechanism, novel targets, between diseases, to be treated by repurposed drugs [60]. This approach has been used to find

matching gene targets already identified for coronary artery disease with information three different drug-target databases (DrugBank, Therapeutic Target Database, and PharmGKB) to select potential repositioning candidates [61]. However, this approach is having certain limitations its utility at present is not much clear [60].

5. Pathway or network mapping

These approaches have been widely used to identify drugs or drug targets for repurposing strategy [62]. This approach gives information about upstream or downstream genes of the GWAS-associated target, which can be thought of having repurposing potential [63]. This involves constructing drug or disease networks based on gene expression patterns, disease pathology, protein interactions, or GWAS or signature matching data to identify the repurposing candidates [64]. This approach helped in identification of 67 common biological pathways having common role in respiratory viral infections [62]. When analyzed against the DrugBank database, these pathways were found with a potential effect against host-viral targets. Pranlukast, a leukotriene receptor 1 antagonist, is one such drug used in asthma and Amrinone, a phosphodiesterase inhibitor, used in the treatment of congestive heart failure that has also been found for repurposing strategy.

6. Predicting drug-target interactions

When a drug binds to protein, it might impact the activity of proteins existing downstream of the target protein. Any side effects, therapeutic mechanisms, or any other novel indications arising upon drugs might help in repurposing it.

6.1 *De novo* structure-based prediction

Based only on drug structure, this approach is useful for virtual screening of large compound libraries. This approach has advantage to provide structural

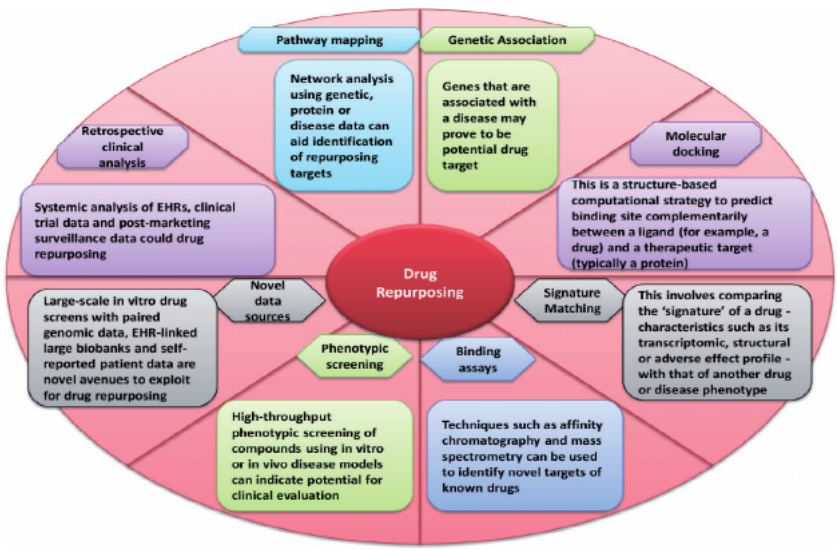

Figure 4.
Approaches (experimental and computational) used in drug repurposing.

insights about the interaction and further guide the optimization the structure to improve the binding affinity for its target. The approach is computationally much demanding, limiting its large-scale use, many-to-many DTI prediction tasks. In a *ligand-based* approach, constructing a "pseudo-drug" representation called a *pharmacophore* model is used for elucidating the interaction with the chosen target [65]. Pharmacophore models can be constructed from analysis of the target's binding pocket, or derived using a set of positive and negative examples of compounds interacting with the target. Compared with molecular docking, this approach is more computationally efficient and has better accuracy [66]. **Figure 4** summarizes various approaches for drug repurposing.

Different computational approaches can be used independently or in amalgamation to systematically analyze different types of large-scale data to obtain significant interpretations for repurposing hypothesis. Experimental approaches can also be used to recognize repurposing opportunities [67]. Computational approaches are mainly data-driven; they involve systematic analysis of data of any type (such as gene expression, chemical structure, genotype or proteomic data, or electronic health records (EHRs)), which can then lead to the formulation of repurposing hypo-theses.

7. *In silico* approach to prioritize drugs for repurposing against TB

FDA-approved drugs are pharmacists' choice pharmacist for repurposing against TB. Bioinformatic approach is economic, time efficient with better chances of success. About 1554 FDA-approved drugs obtained from DrugBank have been approached for TB therapy using *in silico* method. Serine/threonine-protein kinase, pknB (Rv0014c) of *M.tb* was selected as the drug target and all of the 1554 drugs were subjected to molecular docking with pknB. Rigid docking followed by induced fit docking protocol was employed for prioritization of drugs. Fourteen drugs were prioritized, out of which six are suggested as high-confident drugs toward repurposing for TB. These drugs strongly bound in the active site of the pknB. Atorvastatin was one of the high-confident drugs [68]. It has been reported that a gene ontology-based network containing 26,404 edges, 6630 drug, and 4083 target nodes analyzed using network-based inference (NBI) are used to identify novel drug-target interactions that are further evaluated on basis of a combined evidence approach for identification of potential drug repurposing candidates. Targets are prioritized on basis of known variation in clinical isolates and human homologs, essentiality for *M.tb's* survival and virulence. DTIs were used to identify target pairs against which the predicted drugs could have synergistic bactericidal effect. Enlisted DTIs from RepTB, four TB targets, namely, FolP1 (Dihydropteroate synthase), Tmk (Thymidylate kinase), Dut (Deoxyuridine 5′-triphosphate nucleotidohydrolase), and MenB (1,4-dihydroxy-2-naphthoyl-CoA synthase) have the potential for future drug candidature.

7.1 Potential targets for drug repurposing

Information about the structure of drug-binding site reveals novel connections between drugs and targets. A correlation between drug-promiscuity and shared binding sites across the drug's multiple targets demonstrates the potential role of structural analyses of shared binding sites in drug repositioning [69]. A docking-based approach has been employed to screen new novel targets for existing drugs by computationally screening the whole druggable proteome [70]. Target-based screening has revealed potential of anti-Parkinson drugs entacapone and tolcapone against drug-resistant (MDR) and extensively drug-resistant (XDR) TB. The logic

for this activity is based on similarity between the original target COMT and the new target InhA [71]. *M.Tb* phosphoserine phosphatase SerB2 is a promising drug target, being a key essential metabolic enzyme of the pathogen's serine pathway. About one hundred and twenty two compounds from an internal chemolibrary were screened using malachite green-based phosphatase assay and Tri-substituted derivatives were found among the best hits that inhibited SerB2 activity. Their interaction with the enzyme was studied through induced fit docking experiments. Cellular assays showed that the selected compounds also inhibit *M.tb* growth *in vitro*. Those promising results may provide a basis for the development of new anti-mycobacterial agents targeting SerB2 [24]. Drug efflux is an important resistance mechanism in *M.tb.* Different medications used to treat unrelated human conditions such as psychoses and angina serve to inhibit the multidrug efflux pumps in *M. TB;* this increases the pathogen's susceptibility to other drugs. Thiazolidinedione enhances (a) killing of intracellular pathogen by non-killing macrophages and (b) inhibits the expression of efflux pumps that extrude antibiotics prior to their action. The other targets are based on overexpressed efflux pumps, to make otherwise inefficient antibiotics again effective. Molecules 4-OH-OPB depleted flavins-formed covalent adducts with N-acetyl-cysteine and mycothiol. This molecule killed *M.tb* synergistically with oxidants and other anti-TB drugs. The conditions that block *M.tb's* replication modify OPB and enhance its killing action. Modified OPB kills both replicating and non-replicating *M.tb* and sensitizes to both host-derived and medicinal antimycobacterial agents [72]. Several phosphodiesterase inhibitors have also shown promise as adjuvants for host-directed therapy. All phenothiazines are known to have common function to inhibit the binding of calcium to calcium-dependent proteins of eukaryotic cells [73]. Calcium binding is important for the bacterial phagocytosis [74]. Consequently, inhibition of calcium signaling processes, by phenothiazines, ought to affect processes of phagocytosis [75]. Moreover, the killing activity of neutrophils is dependent upon the retention of calcium [76] and potassium within the phagolysosome [77]. Thus, verapamil, an inhibitor of calcium transport, and ouabain, an inhibitor of potassium transport, promotes the killing of intracellular *M. Tb* by non-killing human macrophages [78]. Thiazolidinediones, otherwise an antidiabetic drug, acts as inhibitor of calcium and potassium transport, hence has repurposing potential to promote bug killing [79]. In a study, TDZ treatment to *M. Tb* infected mouse was successful by inhibiting efflux of calcium and potassium from the phagolysosome as potassium is requisite for the phagolysosomal acidification and degradation of the entrapped pathogen. Thiazolidinediones can be sought as future drugs in TB drug repurposing [77]. Increasing resistance to isoniazid due to prolonged exposure of INH-susceptible *M. Tb* strains to increasing concentrations of INH can be reduced to wild-type INH susceptibility by using inhibitors of efflux pump CPZ and reserpine [80]. RIF-resistant *M. Tb*-infected mice have over expression of an efflux pump upon treatment with RIF, rendering the strain resistant to oxacillin as well [81]. Though phenothiazine inhibits the efflux pump systems of mycobacteria [82], only recently has TDZ been shown to inhibit the expression of genes that code for efflux pumps [83]. Specifically, efflux pumps coded by mmpL7, p55, efpA, mmr, Rv1258c, and Rv2459 [84] have direct effects on the efflux pumps of *M. Tb*. An agent that inhibits an efflux pump system, responsible for its resistance to antibiotics renders that organism again susceptible to the otherwise resistant antibiotics [85]. Consequently, when TDZ inhibits the activity of efflux pumps of MDR mycobacteria, it renders the organism susceptible to the antibiotics to which it was initially resistant as a consequence of their extrusion from the cell [86]. However, with time, accumulation of mutations takes place and, commensurate with this accumulation, the level of expression of the efflux pump decreases to almost that of the wild-type parent [86].

Repurposed drugs with synergistic effects: Synergistic effects of repurposed drugs with other anti-TB drugs for treatment of MDR-TB, XDR-TB, and TDR-TB have been proposed for the future WHO regimen. Clofazimine (CZM) in a combination with moxifloxacin (MOX) and ethambutol (EMB) might be a promising drug regimen for the treatment of MDR-TB [87]. Similarly, *in vitro*, synergistic effect of sulfamethoxazole (SMX) has been reported with rifampin [88]. For the treatment of MDR-TB, pyrazinamide and bedaquiline in combination with CZM have been reported as a best example of synergistic effect [89]. The combinatorial therapy of capreomycin and linezolid showed partial synergistic effect suggestive of increased efficacy against *M.tb* [89]. Synergistic therapy of linezolid and bedaquiline has been suggested for rescuing female XDR-TB patients during pregnancy [90]. Synergistic effect of carbapenems is also known with rifampicin against *M. tb* [91]. Thioridazine (TDZ), a neuroleptic drug in combination with antibiotics, kills extremely drug-resistant *M.tb* (XDR-TB). This combination is not prone to mutations as it does not affect the pathogen directly. With proper precautions and cardiac monitoring prior to and during therapy, TDZ will be essentially safe. Given the serious prognoses associated with MDR/XDR-TB and TDR-TB infections, TDZ provides a suitable alternative to current ineffective therapy. Numerous cephalosporins were synergistic with rifampicin, the cornerstone drug for TB therapy and ethambutol, a first-line anti-TB drug. When used in combination, cephalosporins and rifampicin had 4- to 64-fold more activity than used alone. Clavulanate has also shown key synergistic partner role in triple combinations. Cephalosporins (and other beta-lactams) together with clavulanate reversed the inefficacy of rifampicin in a rifampicin-resistant strain. Cephalosporins also showed synergism with new anti-TB drugs such as bedaquiline and delamanid. More studies will be needed to validate their *in vivo* activities. Additional features like oral bioavailability with good safety profiles and antimycobacterial effects of cephalosporins suggest that they could be promising repurposing agents [92]. The newly synthesized and patented SILA compounds were tested for *in vitro* and *ex vivo* activity against XDR-TB. These compounds had *in vitro* activity against XDR-TB (MIC<3.5 mg/L) could transform non-killing macrophages into effective killers of phagocytosed bacteria, without any cytotoxic activity. Among them, SILA 421 revealed good *in vitro* and *ex vivo* activities without exhibiting any cytotoxic activity; thus, it seems to be a potential candidate to be anti-MDR/XDR-TB drug [93].

7.2 Hurdles in TB drug repurposing

Development of *in vitro* models for non-replicating and replicating M.tb Bacilli has not been successfully achieved and presents a big challenge in drug discovery. A multi-stress model of non-replication has been put forward [94]. But interpretation of results using this model is difficult due to involvement of outgrowth period [95]. A rapid method not requiring the outgrowth period has been developed to measure bactericidal activity against non-replicating *Mycobacterium tuberculosis*, induced at low pH (citrate buffer at pH 4.5). It can easily detect viable *M. tuberculosis* strain constitutively expressing luciferase [95]. To establish models that represent real metabolic state in various host niches, and the related effects of micro-biome status, nutritional state, and other underlying health issue like diabetes, a significant success is still a dream. So, no screening model can be sufficient enough to bypass extensive follow-on experiments in the human host to ascertain efficacy, pharmacokinetics, pharmacodynamics, toxicity (e.g., specificity), and the mechanism of action to yield better results with more optimization of molecules using medicinal chemistry. In addition to safety concern of the drug, its interaction with other antimicrobial agents is the critical issue to be addressed as the treatment duration

of the disease is long. Ideally, the new drugs are expected to decrease required treatment durations hence improving patient compliance and treatment outcomes. The co-existence of HIV and TB emphasizes that new lead must be compatible with antiretroviral therapy as well as active against resistant forms of TB [96]. Targeting the drug to the site of infection is very long and eventful process, which often makes the compound unable to reach its target in active state at the requisite MIC value for the pathogen [97]. Orally administered drugs are bound to have certain characteristics features for rendering good efficiency. Stability and solubility at the acidic pH, withstanding the first-pass metabolism, adequate lung permeability, uptake by *M. tuberculosis* to reach the intracellular target(s) and chemical stability and activity under pathophysiological conditions some of the features are required for any drug to be repurposed against TB [97–99]. Common challenges of drug repurposing also affect drug reuse against TB. Optimizing selection criteria of target population to evaluate the expected outcome of the drug are one of major challenges. Any error in subject selection can give unexpected adverse results of drug. For example, thalidomide when prescribed for pregnant women in first trimester for managing morning sickness resulted in amelia and phocomelia [100]. Dosing regimen and route of administration are the two important considerations for repurposing of old drug for new indication. The stability of drug formulation is a challenge while optimizing the drug for a new indication [101]. Different physiologies and multiple drug requirements of different patients arise the threat of unexpected adverse events, which mandate the careful investigation of every response upon drug administration. It becomes essential to have data on drug-drug interactions, pharmacodynamics, and pharmacokinetics of the drug prior to its repurposing.

8. Success stories so far

There are many instances where repurposed drugs have shown successful results in subclinical, preclinical, and clinical levels. Mice could be cured of both drug susceptible and MDR infection mice were given TDZ [79, 102] alone [103] and in combination with INH [104]. This study was extrapolated to non-responsive MDR-TB patients in a Buenos Aires hospital (Argentina) [105] and weeks later, patients got cured of TB. The protocol was then modified and included nonresponsive antibiotics, and out of 12 XDR-TB patients, 10 were pronounced cured of the infection [106]. TDZ was also used by *Udwadia* et al. for the therapy of XDR-TB patients in Mumbai (India) and it was found to improve significantly their quality of life. The subclinical and preclinical success of the drug TDZ, against MDR TB and XDR TB, led to a public call to consider TDZ for therapy of non-responsive MDR/XDR-TB under compassionate basis [107]. Meropenem, in combination with clavulanate, was adjusted with the drug regimen and approved by the European Medicines Agency and the US Food and Drug Administration (FDA) for curing TB in 8 months. FDA-approved antidiabetic drug metformin, was shown to enhance the efficacy of other anti-TB drugs against the drug-resistant tuberculosis [47]. *Reports from* Microbiology and Infectious Diseases at the National Institute of Allergy & Infectious Diseases (NIAID) and Stop TB Partnership new drug working group state that drug resistance has arisen against every currently available tuberculosis drug. Successful treatment for extensively drug-resistant (XDR) cases is less than half of that for drug-susceptible tuberculosis; this makes situation grave and urges for new antibiotics against the global killer. Many compounds in TB-advanced clinical trials were formerly used to treat other infectious diseases/TB, and now, they have been repurposed for the treatment of TB [108–110]. Revival of sulfamethoxazole (SMX) in TB occurred when it was first used to prevent the Pneumocystis jirovecii like infections in HIV/TB patients [111]. In a

Nigerian trial study on patients of HIV-MDR-TB co-infection, efficiency of MDR-TB treatment by TMP/SMX confirmed a significantly shorter time to sputum conversion in these patients [112]. Sulfadiazine, an antileprosy drug, was repurposed in the treatment of MDR-TB and XDR-TB [113] suggesting that sulfadiazine regimen is safe and effective against MDR-TB and TDR-TB treatment [113, 114]. Clofazimine (CZM), an old antileprosy drug, was repurposed for managing the treatment of MDR-TB [110]. CZM is now recommended as a second-line anti-TB drug and used in combination with other anti-TB drugs for the treatment of drug-resistant tuberculosis in 9–12 months. Previous published studies have reported that CZM has good quality efficacy and little toxicity against drug-resistant mycobacterial strains in animal models, which suggested, CZM as a promising anti-TB drug for the management of MDR-TB [111]. Linezolid, an oxazolidinone antibiotic used for the treatment of gram-positive bacterial infections [115], is being potentially repurposed for the treatment of drug resistant TB (MDR-TB and XDR-TB) [116]. But it has been limited by various side effects such as neurotoxicity and hematologic toxicity [90]. Safety of bedaquiline and linezolid drug combination has been evaluated by a case study for XDR-TB and found to be safe in even the late third trimester of pregnancy or pregnant woman. Post-treatment, pregnant woman gave birth to a normal child who grew without fatalities [90]. Minocycline is another anti-leprotic drug [117], which was repurposed in 2008 for managing the treatment of XDR-TB patient in Japan [118]. *In vitro* activity of meropenem combined with clavulanate against XDR strains calls for repurposing the beta-lactams as new anti-TB drugs [119]. Carbapenems have been used successfully as part of salvage therapies for XDR patients, which have to be administered intravenously [120]. Recently, an early bactericidal activity-Phase II (EBA Phase II) clinical trial has validated the promising potential of a carbapenem combined with amoxicillin and clavulanic acid for TB treatment [121]. In a controlled clinical trial in tuberculosis, inhaled IFN-γ was effective. These experiences warrant the continued evaluation of inhaled IFN-γ in human clinical trials [47]. Certain clinical studies are exploring the potential of NSAIDs in TB treatment. NCT02060006 is a Phase 3 trial to identify meloxicam in preventing TB immune reconstitution inflammatory syndrome (IRIS), a serious clinical issue in HIV co-infected TB patients. Phase 2 clinical study (NCT02237365) of aspirin and ibuprofen is an adjunctive treatment for TB meningitis for the treatment of XDR-TB in addition to the standard therapy (NCT02781909). The immune-modulatory function of NSAIDs (etoricoxib) in increasing the protection offered when administered alongside a TB vaccine is being investigated in the trial NCT02503839. Other drug-screening study revealed carprofen, an NSAID, to selectively inhibit the growth of replicating, non-replicating, and MDR clinical isolates of *M. tuberculosis* at 40 mg/L [122, 123].

Hurdles in TB drug repurposing: Upon entering and infecting the host, *M.tb* spreads to different micro-niches and evolves as heterogeneous population. To eliminate each physiological state of the bug, any new anti-TB needs to be active under these conditions [124]. Development of *in vitro* models for non-replicating and replicating Bacilli has not been successfully achieved. Subpopulations of non-replicating bacilli have present inside host arise need for the lengthy anti TB drug therapy and turn out to be reservoir from which drug-resistant bacteria emerge [125]. A multi-stress model of non-replication has been put forward [94]. But a disadvantage of this type of model is the need for a recovery or outgrowth phase that implies bacilli being replicated, which makes interpretation more difficult [33]. A rapid method has been developed to measure bactericidal activity against non-replicating *M. tuberculosis*, without requirement of the outgrowth period, and easily detecting luminescence of viable *M. tuberculosis* strain constitutively expressing luciferase [95]. Compounds with bactericidal activity against non-replicating bacteria were identified employing a pH-sensitive green fluorescence protein screening

approach devised to identify compounds that disrupt the ability of *M. tuberculosis* to maintain its internal pH in an acidic environment [126]. Since TB is a complex disease, no *in vitro* model has been till date established to predict *in vivo* efficacy [127]. Tuberculosis is the leading cause of death from infectious disease. Current drug therapy requires a combination of antibiotics taken over >6 months. An urgent need for new agents that can shorten therapy is required. To develop new drugs, simple *in vitro* assays are required that can identify efficacious compounds rapidly and predict *in vivo* activity in the human. Areas covered: This review focusses on the most relevant *in vitro* assays that can be utilized in a drug discovery program, which mimics different aspects of infection or disease. The focus is largely on assays used to test >1000s of compounds reliably and robustly. However, some assays used for 10s to 100 s of compounds are included where the utility outweighs the low capacity. Literature searches for high-throughput screening, models, and *in vitro* assays were undertaken. Expert opinion: drug discovery and development in tuberculosis is extremely challenging due to the requirement for predicting drug efficacy in a disease with complex pathology in which bacteria exist in heterogeneous states in inaccessible locations. A combination of assays can be used to determine profiles against replicating, non-replicating, intracellular, and tolerant bacteria [127]. To establish best representative model of the real metabolic state, either replicating or non-replicating bug in various environments inside human host is a challenge. Screening models fail to fulfill requirements of extensive follow-on experiments in the human host to ascertain efficacy, pharmacokinetics, pharmacodynamics, and toxicity, and thus hamper the optimization for improvement of repurposed drug efficacy using medicinal chemistry approach. In addition, safety concern of the drug and its interaction with other antimicrobial agents are the critical issues to be addressed as the treatment duration of the disease is long. Ideally, the new drugs are expected to decrease required treatment durations hence improving patient compliance and treatment outcomes. The co-existence of HIV and TB emphasizes that new lead must be compatible with antiretroviral therapy as well as active against resistant forms of TB [96]. Targeting the drug to the site of infection is very long and eventful process, which often makes the compound unable to reach its target in active state. A drug molecule has to travel from the blood circulation to non-vascularized pulmonary lesions wherefrom it shall diffuse into necrotic foci and the caseum of granuloma and then permeate the lipid-rich cell envelope of bacilli at the requisite MIC value for the pathogen [97]. Common challenges of drug repurposing also affect drug reuse against TB. Optimizing selection criteria of target population to evaluate the expected outcome of the drug is one of major challenges. Any error in subject selection can give unexpected adverse results of drug. For example, Thalidomide when prescribed for pregnant women in first trimester for managing morning sickness resulted in amelia and phocomelia [100]. Repurposing of old drug for new indication needs addressing the dosing regimen and route of administration to yield the considerable benefits against new target. Patient-specific repurposing of drug shall be aimed to evade the adverse events, which might occur due to differential response of different patients to the repurposed drug. Moreover, prerequisite data on drug-drug interactions, pharmacodynamics, and pharmacokinetics of the drug shall be keenly studied prior to further studies of the drug.

9. Status of TB drug repurposing and its future perspective

Drug repurposing is undoubtedly an alluring strategy to develop a new treatment regimen for tuberculosis within a short span of time and also to treat and curb drug-resistant pathogens [128]. Few of the repurposed drugs have shown great potential

for future treatment of TB and have been extensively studied. Nevertheless, the incidence of resistance in the *M.tb* population is occurring at a very fast rate and therefore, we urgently need a new improved treatment regime via repurposing many drugs using various approaches such as experimental and computational biology [129, 130] to scrutinize the potential of already existing thousands of drugs to minimize the time required for novel drug discovery. Organization of such studies is on the human cohorts, as the influence of the host-protective immune system continues to gain attention in the advancement of host-directed therapies, so effect of repurposed drugs on the balance of the host immune system, infection, and inflammation shall be explored. This will update concepts to design combinational therapies to shorten the treatment regime and preventing drug resistance while being cost effective and safe for general masses [131]. Repurposing drugs assuredly provide an appealing strategy in the process of modern drug development and exceptionally/especially against tuberculosis, which already have numerous engrossing old drugs with *in vitro* growth inhibitory activities. Using different methods for whole-cell evaluations such as HT-SPOTi [132, 133] and micro-plate Alamar blue assays (MABA) [134] has turn out to be crucial for the expeditious detection of various old drugs that have promising potential in drug repurposing. Many of the potential anti-TB drugs were identified through serendipity, and amalgamating the various assays with systems biology will in turn provide a reasonable approach in the identification of these drugs [135]. TB drug discovery paradigm converses from the conventional one-target one-drug to a multi-target multidrug scheme, and various potential drugs for repurposing are being recognized and put forth into the advanced phases of clinical trials. As an alternative in the treatment of drug-resistance, repurposed drugs have already proven their potential and effectiveness. Endeavor to repurpose inexpensive, safe and universally available drugs should continue to deliver the anti-TB therapies required by many who would not otherwise have access to a cure [128]. On one side, it becomes imperious to find new candidate drugs to control TB, and on the other side, it is also important to continuously redefine, revise, reclassify, and perhaps, repurpose drugs that are already in use. The drug repurposing offers manifold advantages. It is therefore pivotal to understand their secondary targets and various endogenous molecular mechanisms of action and its translation into a multidrug combinatorial treatment regimen. Identification of mechanism of action of these repurposed drugs will definitely strengthen their inclusion in clinical trials and gravel the way for designing more targeted drugs. As antimicrobial resistance deepens, the search to find novel drugs and to evaluate the mechanism of resistance would widen our search to novel concepts as well to find a better cure to curb TB than what already exists. Repositioning of pre-existing drugs seems to be a strategy to avoid enormous investment in funds and time. Drugs with already known toxicity and safety profiles have been screened against the TB pathogen and found to be effective against various physiological states of pathogen. The endogenous targets of these drugs against *M.tb* are likely to be novel; thus, minimal chances of resistance arise. Moreover, few of these drugs may have multiple targets, which indicate minimal development of resistance. Thus, repurposing the pre-existing molecules offers colossal/enormous potential to tackle extensively drug-resistant TB infections. Fluoroquinolones prevent DNA replication by inhibiting topoisomerase II and IV; two examples *viz.* gatifloxacin and moxifloxacin are active against *M. tuberculosis* both *in vitro* and *in vivo* conditions [136, 137] and thus used as second-line drugs against TB [138]. Moxifloxacin was advanced to phase III clinical trials to evaluate its potential to shorten the duration of conventional TB therapy (**Figure 5**) [139, 140].

Schematic illustration of the *Mycobacterium tuberculosis* cell membrane includes the electron transport chain (ETC), efflux pumps (EPs) and the site of action of

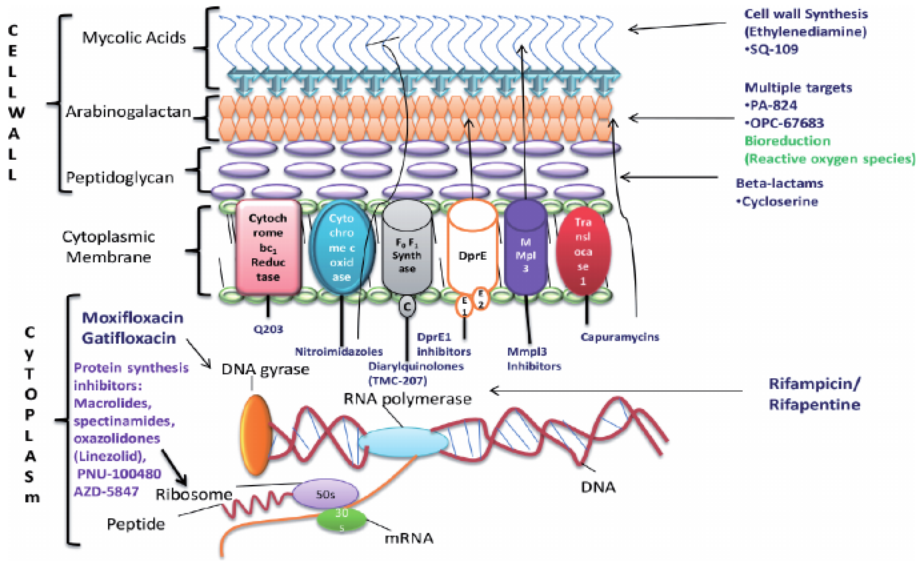

Figure 5.
Mechanism of action of new anti-TB agents in different stages of clinical drug development pipeline for tuberculosis.

several antituberculosis drugs. By damaging the cell membrane, the lipophilic drugs will affect the activity of several membrane enzymes such as those involved in the ETC and efflux pumps responsible for the extrusion of several compounds from the cell. The inhibition of any component of the ETC reduces energy production and disrupts membrane potential. Consequently, the disruption of the PMF reduces the activity of the efflux pumps. SQ-109 has been reported to act by inhibiting the mycobacterial trehalose monomycolate transporter MmpL3, involved in cell wall biosynthesis [141]. PA-824 is effective not only toward the actively replicating but also against the non-replicating bacteria. They inhibit the synthesis of mycolic acids and induce respiratory poisoning [142] Q-203 targets the Cytochrome b subunit (QcrB) of the cytochrome bc1 complex (complex III), which is an essential component of the *M. tuberculosis* respiratory electron transport chain, forcing *M. tuberculosis* to use the cytochrome bd, a terminal oxidase energetically less efficient [143]. Q-203 causes a rapid depletion of the intracellular ATP levels at 1.1 nM and is able to interfere with ATP homeostasis in nonreplicating *M. tuberculosis* at concentrations of <10 nM, suggesting the inhibition of cytochrome bc1 activity as its primary mode of action [144]. Diarylquinolines target subunit c of mycobacterial ATP synthase [145]. Mycobacterial membrane protein large (MmpL) proteins, which belong to the resistance, nodulation and cell division (RND) superfamily of transporters, play a central role in shuttling lipid components to the cell wall. These transporters work with accessory proteins to translocate virulence-associated envelope lipids and siderophores across the inner membrane [146]. Capuramycin and its analogs are strong translocase I (MurX/MraY) inhibitors [147]. Oxazolidinones inhibit the initiation of protein synthesis by preventing the formation of the $tRNA^{fMet}$-mRNA-70S (or 30S) subunit ternary complex [148].

It is under evaluation in a TB Alliance phase III clinical trial with pretomanid and pyrazinamide (PaMZ). Mycobacterial resistance to fluoroquinolones is evident [149] caused by stepwise mutations in the target genes such as gyrA and gyrB [150]. There is no visible cross-resistance observed with the other first-line drugs [151], but there is cross-resistance within this group of molecules. Indeed, this cross-resistance is not universal [152], and newer fluoroquinolones such as TBK613 will still be effective

against fluoroquinolone-resistant strains. This demonstrates the coherent nature of the development of novel drug and drug repositioning, and structure-activity relationship of a repurposed drug enables the design of novel molecules with higher potency. Nitroimidazopyrans, resembling the antibiotic metronidazole, is active against actively growing and dormant *M. tuberculosis* [33, 153]. The novel chemical entities (NCEs) OPC-67683 and PA-824 are currently in clinical trials [154]. Metronidazole is also highly active against *M. tuberculosis* [33] and has been reported to prevent the reactivation of dormant bacilli in macaque infection models [155]. Clavulanate, a β-lactamase inhibitor, in concurrence with carbapenems showed killing of *M. tuberculosis in vitro* [156] and in a murine TB model [157]. β-lactam tebipenem, originally developed to tackle respiratory and otolaryngological infections in pediatric patients [158], is to be the most potent anti-TB oral carbapenem in combination with clavulanic acid, and clinical trials may start soon. Clofazimine, the antileprosy drug with promising candidate to get repurposed in treating incidences of multidrug-resistant (MDR)- and XDR-TB, is listed as a World Health Organization recommended second-line drug. Members of the avermectin family, traditionally used as antihelminthic agents, have been found to inhibit the growth of even MDR strains of *M. tuberculosis in vitro* [159]. Nitazoxanide has been found to inhibit both replicating and non-replicating forms of *M. tuberculosis* [160, 161]. Disulfiram inhibited *M. tuberculosis* H37Rv growth at a concentration of 5.26 mM [162]. Disulfarim showed the same level of inhibition against clinical isolates and MDR and XDR strains, and an *in vivo* experiment on guinea pigs demonstrated astonishing bactericidal activity [162]. Non-steroidal anti-inflammatory drugs (NSAIDs), oxyphenbutazone [72], and carprofen [163] inhibited the growth of *M. tuberculosis* H37Rv at micromolar concentrations. To develop novel TB treatments, drug repurposing has procured acceptance and has gained pace, with various drugs that are already at different phases of preclinical and clinical trials (**Table 1**) (**Figure 6**) [123].

The drugs and their targets are highlighted in lighter and darker shaded boxes, respectively. The anagram MAGP is used to indicate the "mycolic acid–arabinogalactan–peptidoglycan" layer of the mycobacterial cell wall and PBP refers to the penicillin-binding proteins responsible for the maturation of the cell wall peptidoglycan [177] inhibition of efflux pumps by Thioridazine [178] Fluoroquinolones (moxifloxacin, gatifloxacin), with target of gyrase, are among the drugs used to treat tuberculosis [179]. Oxazolidinones: (Linezolid) kills *Mycobacterium tuberculosis* by binding and blocking tRNA in the peptidyltransferase center (PTC) on the 50Sribosomal subunit, which includes the 5SrRNA and 23S rRNA [180]. Nitroimidazole derivatives: (Metronidazole) with lower reduction potential can selectively tap into the redox system of the microbe (as opposed to mammals) and produce bactericidal activity specific to the microbe [154]. The combination of clavulanate with β-lactams, especially meropenem, was also tested for the ability to inhibit the growth of extensively drug-resistant (XDR) clinical strains of *M. tuberculosis* [119]. *Ibuprofen (IBF) and carprofen, two non-steroidal anti-inflammatory drugs currently used as pain relievers in humans and animals, respectively, displayed specific growth inhibitory properties against the M. tuberculosis complex.* IBP showed antitubercular properties, while carprofen was the most potent among the 2-arylpropanoic class. On the basis of the human targets of the 2-arylpropanoic analgesics, the protein initiation factor infB (Rv2839c) of *M tuberculosis* was proposed as a potential molecular target [163].

Entacapone and tolcapone inhibit enoyl-acyl carrier protein reductase (InhA) [71], which is important component in the synthesis of long-chain mycolic acids. Entacapone and tolcapone are not prodrugs like isoniazid and do not require enzymatic activation. Thus, the primary mutations in enzyme causing resistance

Name	Class	Current use	*In vitro* MIC against H37Rv	Stage of repurposing	References
Clofazimine	Riminophenazine	Antileprosy	1.6 μM	NC003 (phase IIa)—complete; results in 2014. Second-line treatment for TB	[164]
Carprofen*	2-Arylpropanoid acid NSAID	Analgesic	146 μM	Anti-TB property detected *in vitro* by HT-SPOTi	[163]
Chlorpromazine*	Phenothiazine	Antipsychotic	47 μM	Mouse model studies using MDR-TB strains	[165]
Disulfiram*	Thiocarbamate	Alcohol withdrawal drug	5.3 μM	Anti-TB property detected by broth dilution tests	[162]
Ivermectin	Avermectin	Antihelminthic	6.8 μM	Anti-TB property detected by MTT assay	[159]
Entacapone	Nitrocatechol	Anti-Parkinson's drug	205 μM	Anti-TB property predicted by systems biology. *In vitro* activity detected by broth dilution	[166]
Gatifloxacin	Fluoroquinolone	Respiratory infections	660 nM	Phase III; enrolment complete	[167]
Linezolid	Oxazolidinone	Gram-positive bacteria	741 nM	Phase II completed	[168]
Metronidazole	Nitroimidazole	Broad-spectrum antibiotic	>1.4 mM	Phase II completed	[153]
Meropenem/clavulanic acid	β-Lactams	Antibiotic	1.7 μM	*In vivo* and small-scale human patient studies	[169, 170]
Moxifloxacin	Fluoroquinolone	Acute bacterial sinusitis	1.1 μM	REMox TB—completed STAND (phase III)—enrolment begins in 2014	[171]
Nitazoxanide	Nitrothiazole	Antiprotozoal	52 μM	*In vitro* activity detected	[161]
Oxyphenbutazone*	Pyrazolidinedione NSAID	Analgesic	200 μM (12.5 μM against non-replicant)	*In vitro* activity detected	[72]
Pyrvinium pamoate	Methylquinolinium	Antihelminthic	310 nM	*In vitro* activity detected by Alamar blue assay	[172]
Tebipenem/clavulanic acid	β-Lactams	Antibiotic	2.9 μM	Enzyme inhibition studies	[156, 173]

Name	Class	Current use	*In vitro* MIC against H37Rv	Stage of repurposing	References
Thioridazine	Phenothiazine	Antipsychotic	27 μM	Anti-TB property detected *in vitro* by BACTEC 460-TB	[79]
Tolcapone	Nitrocatechol	Anti-Parkinson's drug	457 μM	Anti-TB property predicted by system biology	[174]

Table 1.
List of drugs in progress for repositioning against TB; given their original indication. Drugs marked with an asterisk (*) are probable candidates for inclusion in TB treatment regimens as host-directed adjuvant therapy due to their immune-modulatory activity.

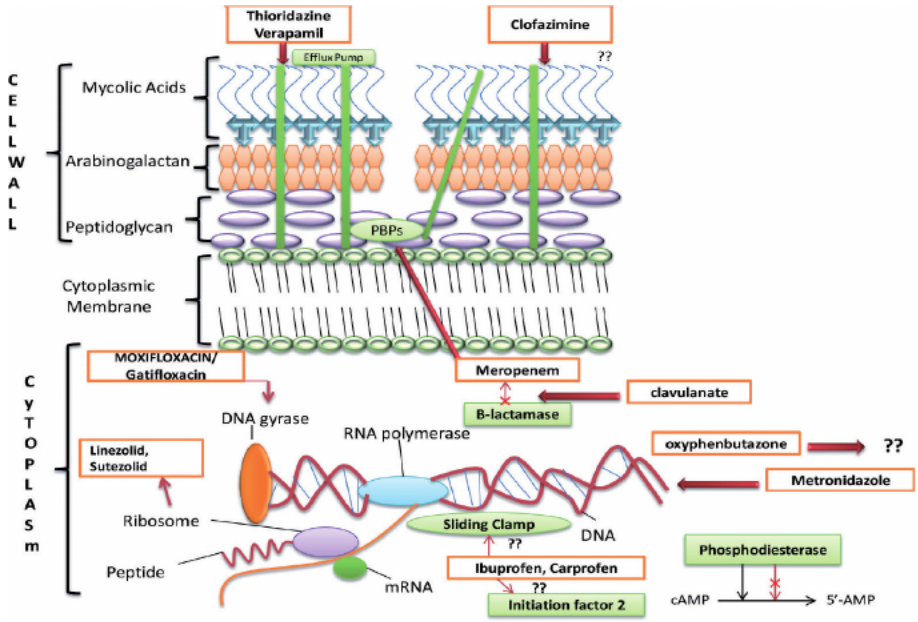

Figure 6.
The possible endogenous mechanisms of action of repurposed drugs, and many anti-infectives previously used for other disease indications are being considered for, or are already in various phases of in vitro/in vivo, as well as advanced clinical trial studies [175, 176].

could be avoided as the resistant mutation in the activating catalase KatG, which is being exhibited by many MDR strains. Chlorpromazine and thioridazine are the members of the phenothiazine class of neuroleptics, and both have been found to inhibit the growth of mycobacteria [79, 166]. Non-steroidal anti-inflammatory drugs (NSAIDs) have been already acclaimed for their anti-inflammatory effects but their antibiotic potential needs further exploration. Structural modifications to improve the antimicrobial activities of NSAIDs such as ibuprofen and carprofen are already ongoing [181]. On the basis of active pharmacophore of celecoxib, analogs that show potent inhibitory activity against *M. tuberculosis* and *S. aureus* have been synthesized and further efforts to optimize these compounds are in progress [182]. The role of aspirin in combination with corticosteroids against TB meningitis has shown to decrease the incidence of strokes and mortality [183]. In a TB treatment, NSAIDs are principally used to mitigate the symptoms that arise from the effects of this prolonged disease and its therapy. In basic animal models, these compounds have already proven pharmacokinetic/dynamic and toxicity profiles, as such there is rational evidence to justify their admittance into early clinical trials. However, the stage of disease and route of administration needs critical consideration for further setting a clinical trial [177]. Compounds with ability to activate or suppress immune system are called immuno-modulators and may be natural or synthesized in origin. These compounds either release pro-inflammatory or anti-inflammatory cytokines to improve the immune response for the efficient killing of the pathogen [184]. To initiate this cascade of events, the pro-inflammatory cytokines are responsible. The immune-modulators act on different immune cells such as lymphocytes, neutrophils, macrophages, natural killer (NK) cells to exert their effector responses aimed at clearing the bacteria from the host. Upon being administered together with the DOTS, immuno-modulators help in the early clearance of the infection and in the prevention of drug-resistance [131]. Some immune-modulators also help in preventing the side effects of the harsh anti-TB antibiotic therapy. WHO has

recommended the inclusion of repurposed drugs such as clofazimine, carbapenems, fluoroquinolones, and linezolid, among many others, for the treatment of drug-resistant TB. Among these, clofazimine, being used as part of anti MDR-regimen, is inexpensive and carries a promising ability to be a future TB drug [185]. The pravastatin and statin are still in Phase 2b clinical trials after more than two decades of research on their use as anti-TB agent [186]. But the promising results in mice models motivate to go for further clinical trials. Diclofenac, mainly used to treat arthritis and gout, has recently been used as an antimicrobial drug by *Dutta et al*. and showed its treatment reduced bacterial burden and disease pathogenesis in mice as compared with the control group [187]. Diclofenac also exhibits synergy with streptomycin in mice model of TB [188]. Ibuprofen, like indomethacin, is an undiscriminating –COX inhibitor. Ibuprofen has been reported to promote survival of *M.tb* infected mice while decreasing the number and size of lung lesions because of the low bacterial burden [189]. Byrne et al. have further confirmed that both aspirin and ibuprofen help to shorten the Tb treatment course when used along with the first-line anti-TB drugs [190]. Fluoroquinolones, though well known to exert anti-inflammatory functions, have not been much explored for their immune-modulatory properties in TB. Verampil has shown promising results against TB but there is not sufficient literature study on the effect of verapamil on the immune system. Thus further study is to establish the role of verapamil as an immune-modulator in TB. Significant reduction in the mortality rate in patients receiving both metformin and DOTs treatment has been reported [191, 192]. Metformin affects the number of total white blood cells and neutrophils and with an increase in the ratio of monocytes to lymphocytes in the circulation [193]. Diacon et al. have reported the combinatorial use of amoxicillin/clavulanic acid with carbapenems reduces the *M.tb* burden [194]. But there are scarce reports on the immunological aspects related to the compounds. Therefore, further research is needed for successful repurposing of the drug as antitubercular drug. Sulfadiazine, a leprosy-drug, has been repurposed to treat DR-TB and found to be more efficacious and safe than other anti-TB sulfa drugs [113, 195]. To include such drugs in TB treatment, more trials shall be conducted using random human cohorts as subjects. **Table 1** enlists the drugs in progress for repositioning against TB.

On the basis of reported literature based on bioinformatics, proteomics, and repurposing/repositioning/revival of drugs, it is estimated that bioinformatics and proteomics play a pivotal role in the exploration of diagnostics, therapeutics, and mechanism of resistance against drug resistance tuberculosis. Repurposing is a strategy to handle the grave situation of drug resistance tuberculosis in this era of growing antibiotic resistance. Synergistic effect of repurposed drugs along with the newer anti-TB drugs (bedaquiline and delamanid) is a rising hope for the treatment of MDR-TB, XDR-TB, and TDR-TB.

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Drug Repurposing for Tuberculosis

Nicole C. Cardoso, Carel B. Oosthuizen, Nashied Peton and Vinayak Singh

Abstract

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a major global health concern given the increase in multiple forms of drug-resistant TB. This underscores the importance of a continuous pipeline of new anti-TB agents. From recent studies, it is evident that the increase in drug efficacy is being achieved through re-engi-neering old TB-drug families and repurposing known drugs. This approach has led to producing a newer class of compounds which not only saves time and investment in developing newer drugs but is also effective in identifying drug candidates with novel mechanisms to treat multi-drug resistant strains. The repurposed drugs moxi-floxacin, linezolid, and clofazimine are used to treat extensively drug-resistant TB when first- and/or second-line drugs fail. The Chapter covers a detailed background on the current status of the repurposed drugs in the TB drug-discovery pipeline and discusses a potential way forward.

Keywords: tuberculosis, repurposed drugs, drug discovery pipe-line, *Mycobacterium tuberculosis*

1. Introduction

Highlights

- Within TB drug discovery, drug repurposing is a growing field and has established several viable candidates from 'old' drugs for further investigation.
- Drug repurposing for TB could improve therapeutic interventions in low to middle income countries and is an ideal approach due to the saving of time, effort, and most importantly, money.
- The use of computational techniques, including virtual screening of known drugs, have been shown to accelerate the process.
- This approach has the potential to lead to the identification of novel drug targets in *M. tuberculosis*, which could initiate new target-based discovery programs.

Tuberculosis (TB) has been, and continues to be a global health threat, and remains the leading cause of death due to a single infectious agent (*M. tuberculosis*), having claimed ~1.4 million lives in 2019 alone [1]. In the past 2 years, the Covid-19 pandemic has further exacerbated the threat of TB mainly due to a decrease in TB case detection, with trajectories predicting an increase of ~1 million additional

new cases per year from 2020 to 2025 [1]. Furthermore, considering the increasing prevalence of drug resistant (DR) (Rif resistant-RR, multidrug resistant-MDR, and extensively drug resistant- XDR) forms of TB infections, the need for more effective treatment strategies has not been direr. The current standard treatment regimen for drug-susceptible (DS) TB has been in use for decades and includes a combination of four drugs: isoniazid (Inh), rifampicin (Rif), ethambutol (Emb) and pyrazinamide (Pza) for 2 months and a further 4 months of only Inh and Rif (**Figure 1** [2, 3]). The treatment of DR-TB is more complicated and can take up to 18 months, depending on the resistance profile of the infection. Although available, several challenges are faced during the treatment of TB disease. Most notable is the duration and complexity of treatment, toxicity and in the case of HIV-TB coinfection, the possible adverse interactions between anti-TB drugs and antiretrovirals. Despite these challenges, treatment success rates of 85% and 57% have been reported for DS- and DR-TB respectively in 2019 [1]; however, these will not be sufficient to meet the milestones setup as part of the End TB Strategy which include a 90% reduction in incidence rates and 95% reduction in mortality by 2035 compared to 2015 [4]. Optimization and implementation of innovative tools including new drug and treatment regimens are predicted to significantly improve this outlook.

The past 20 years have seen considerable progress in the TB drug discovery arena, with 13 new compounds currently in clinical trials (https://www.newtbdrugs.org/pipeline/clinical). The highlights of TB drug discovery include Bedaquiline (Bdq), Delamanid and, most recently, Pretomanid (PA-824). Within the last 9 years, these were the first three new drugs to be approved for the treatment of TB since the discovery of Rif in the 1960's. Although currently only approved for the treatment of

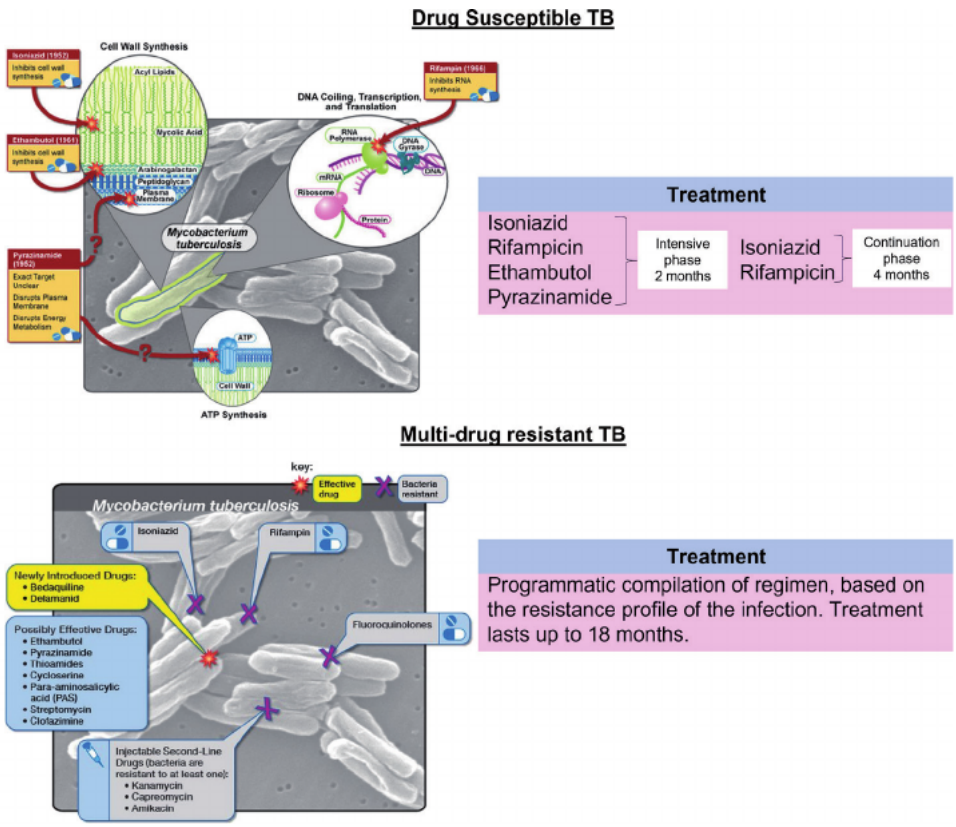

Figure 1.
Current drugs used for the treatment of TB. Adapted from [2, 3] (CC BY 2.0).

DR-TB, both Bdq and PA-824 are being tested as part of novel combination regimens for the treatment of DS-TB. Further highlighting the progress of the TB drug discovery field, the pre-clinical pipeline is also rich in new compounds.

The current scope of the drug discovery and development pipeline is promising; however, the development of a novel drug is a complicated, laborious, and expensive endeavour. From initial screening to clinical usage, the development of a new compound can take up to 15 years and cost more than $1 billion (**Figure 2**) [5, 6]. In addition, there is a high attrition rate of hit compounds during the discovery cascade and clinical trials, further adding to the difficulty of getting novel antimicrobials into the clinic [5–7]. To overcome some of the challenges faced during conventional drug discovery programs, a strategy that has been gaining more interest in recent years is "Drug Repurposing".

Drug repurposing is the process of identifying novel uses of existing drugs for the treatment of disease outside of the scope of the original medical indication. It is also referred to as drug repositioning, redirecting, re-tasking, reprofiling or recycling [8, 9]. This strategy offers several advantages over a conventional drug discovery approach, including (i) reduced risk of failure, (ii) quicker development times, (iii) less investment and lower average costs, and (iv) the possibility of identifying new targets and/or pathways for further investigation (**Figure 2**) [8–10]. Drug repurposing has been successfully applied to several diseases and conditions including HIV, cancer and arthritis [9]. While offering notable advantages over a conventional approach, candidate compounds discovered via drug repurposing are still subject to regulatory requirements prior to therapeutic implementation. These requirements include compound acquisition and licencing, development/optimization for the new application via clinical trials and registration with the relevant regulatory bodies (**Figure 2**).

Repurposing is not new to the treatment of TB. The backbone of the current regimen, Rif, belongs to the rifamycin group of antibiotics [11]. Rifamycins were originally developed for broad-spectrum antibacterial activity and through structure–activity relationship studies, was shown to have the greatest growth inhibitory effect against mycobacteria [11, 12]. The mechanism of action (MoA) of rifamycins involves the inhibition of DNA-dependent RNA polymerase, thus interfering with

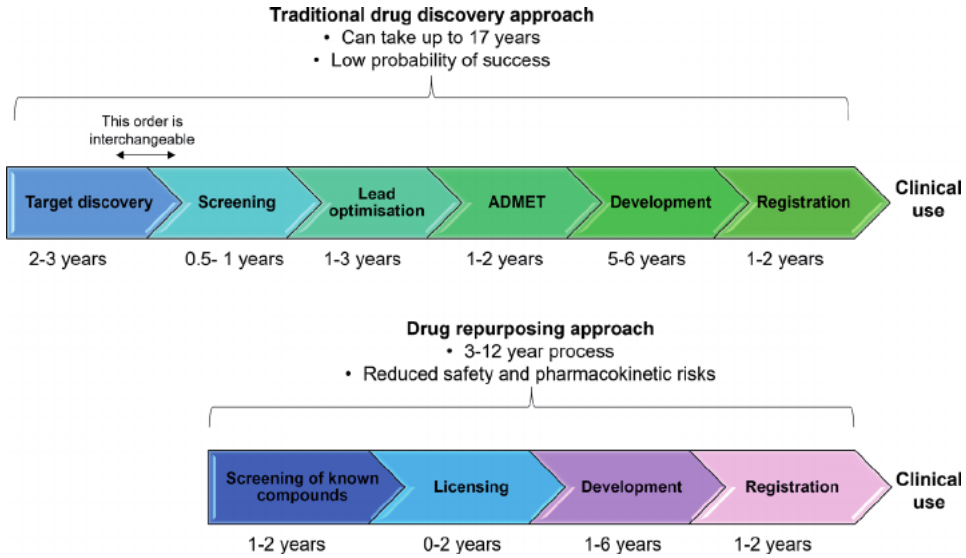

Figure 2.
A comparison of the time taken to get into the clinic when using a traditional drug discovery approach versus a drug repurposing approach. ADMET: Absorption, distribution, metabolism, excretion and toxicity. Adapted from [5].

transcription. While the main application is for DS-TB, Rif has also been used for other bacterial infections e.g. treatment of staphylococcal endocarditis, eradication of group A beta-hemolytic streptococci from pharyngeal carriages and as prophylaxis for close contacts of paediatric patients with *Haemophilus influenzae* or *Neisseria meningitidis* infections [13]. In recent years, drug repurposing has once again gained traction for novel TB treatments, evidenced by 6 different repurposed drugs currently being evaluated in Phase II or III clinical trials [1]. Following an analysis of the published literature related to drug repurposing for TB, the repurposed drugs that are currently in the pre-clinical and clinical pipeline, their molecular mechanisms and therapeutic applications will be discussed further.

2. State of the art

In order to assess what the current scientific field entails, a network analysis was conducted from the Web of Science database (All Databases) using the search terms: repurpose* (repurposed, repurposing), tuberculosis and drug* (drugs). A total of 424 publications were identified within the search criteria and it is evident from **Figure 3** that there has been an increase in research involved with the repurposing of old drugs in the fight against TB. In 2020, 77 manuscripts were published related to this topic, and this is expected to further increase in 2021. Additionally, VOS viewer, was used to assess specific keywords within the total number of publications (https://www.vosviewer.com/). The co-occurrences of all keywords were counted using a full counting method. The minimum keyword occurrence was set to three and out of the 416 identified keywords, 35 met the selection criteria. The third most occurring keyword, after "*M. tuberculosis*" and "Tuberculosis", was "*in vitro*", which indicates that this field of enquiry is still at an early stage (**Figure 4**). This is reiterated by the increase in publications on repurposing in recent years (**Figure 3**) as well as the identification of "drug repositioning" in **Figure 5**. Interestingly, the only drug that satisfied the selection criteria was thioridazine, an antipsychotic drug. It would be expected that additional repurposed drugs will occupy this space as more data becomes available and clinical trials are completed.

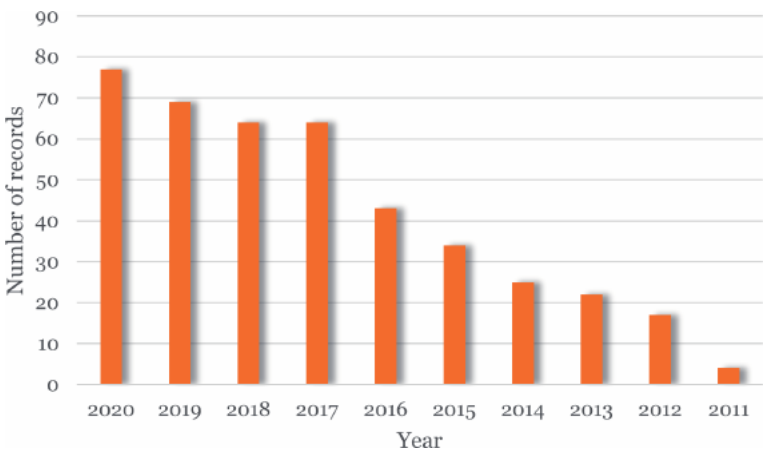

Figure 3.
A steady incline in recent years of the number of scientific articles, related to the search topic "repurposing drugs for tuberculosis". The bars represent the number of published articles according to year. The year 2020 accounts for 18.2% of the published articles related to this topic. (web of science (https://www.webofknowledge.com)).

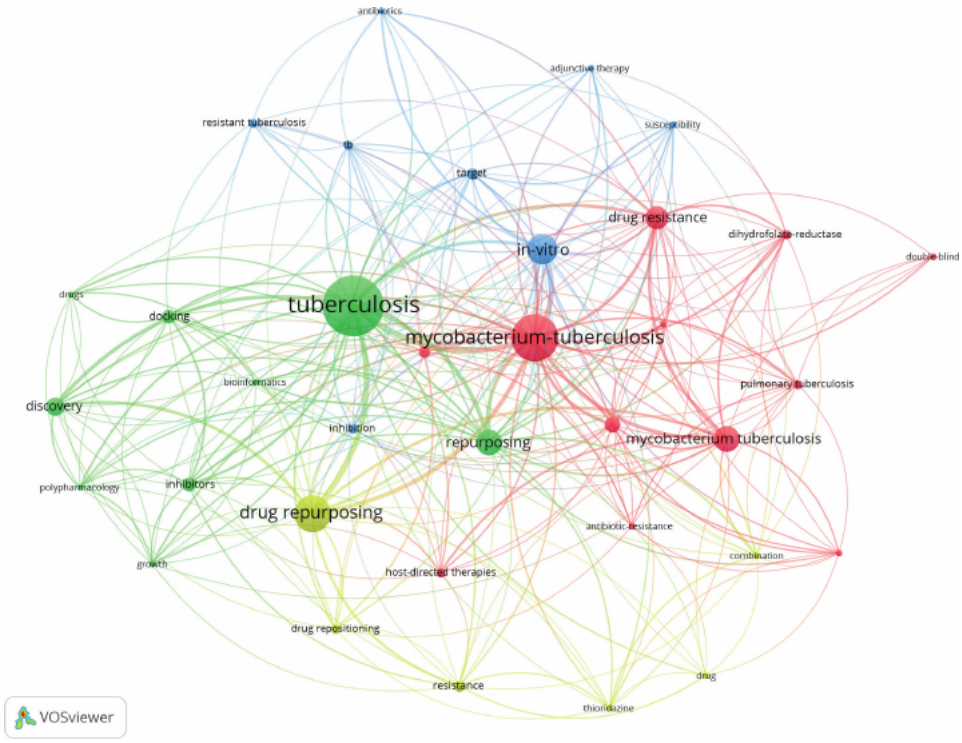

Figure 4.
Bibliographic network analysis of the keywords in published scientific articles, using the search terms "repurposing drugs for tuberculosis" (web of science – All databases). The circles indicate 35 of the most re-occurring keywords, while the size of the circles represents the importance of the keyword. The lines represent the interconnectivity of the keywords (www.vosviewer.com).

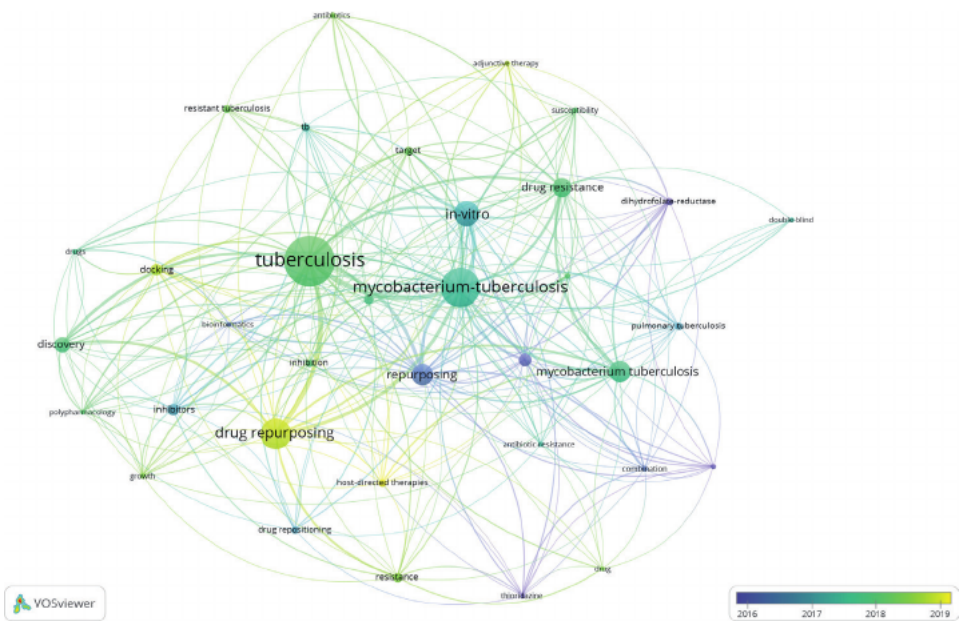

Figure 5.
A time-correlation analysis of the published material related to the search terms. An increase in articles mentioning "drug repurposing", "host-directed therapies" and "adjunctive therapy" can be seen. A trend towards computational approaches, including "docking" is also evident (www.vosviewer.com).

3. Repurposed drugs in the clinical development pipeline

There are approximately thirty chemical compounds currently being investigated in the global TB drug pipeline, of which 15 are classified as repurposed and will be discussed further.

3.1 Linezolid, Sutezolid, Delpazolid and TBI-223

Linezolid, also known as Zyvox, is a first-generation oxazolidinones which are a class of antibiotics that inhibits bacterial protein synthesis. Linezolid works by binding to a site on the bacterial ribosome thereby preventing the formation of a functional 70S ribosomal unit which is an essential component of the bacterial translation process [14–17]. Linezolid was initially approved for the treatment of infections originating from Gram-positive bacteria and used primarily in the treatment of complicated skin infections such as methicillin-resistant *Staphylococcus aureus* (MRSA). Although linezolid exhibits good antimycobacterial properties, its use is limited to DR-TB as its long term toxicity profile have been associated with neurological disorders resulting from nerve damage as well as immunosuppression resulting from decreased production of vital immune cells required for host defence [16, 17]. Analogues of Linezolid namely Sutezolid, Delpazolid, Posizolid, Contezolid and TBI-223 are second-generation oxazolidinones that are showing promising potential as antimycobacterial agents. This is due to enhanced safety profiles and reduced toxicity compared to Linezolid as well as more potent activity against mycobacteria *in vitro*. Studies and clinical trials for these analogues are ongoing with the hopes that they may also be effective in shortening current TB treatment regimens [16, 18, 19].

3.2 Moxifloxacin, Gatifloxacin, levofloxacin and DC-159a

Moxifloxacin and Gatifloxacin are fourth-generation broad-spectrum antibiotics belonging to the family of fluoroquinolone drugs. The main function of this class of antimicrobials is to inhibit the bacterial enzymes DNA gyrase and topoisomerase IV which are crucial for DNA duplication events such as transcription, recombination and cell replication [16, 18, 19]. They were initially approved for the treatment of a number of bacterial infections of the skin, stomach and lungs and along with levofloxacin has also shown promise as an effective and safe candidate for inclusion in the current TB treatment regimen [16, 20]. This is mainly because of their potent antimycobacterial activity as studies have shown that they can significantly improve sputum culture conversion rate and clinical outcome of TB treatment as well as reduce TB resurgence after treatment [17, 21]. These antimicrobials are currently being evaluated as a possible replacement for Isoniazid or Ethambutol in patients with poor tolerability as they were shown to exhibit potent antimycobacterial activity *in vitro* [16]. Moxifloxacin, Gatifloxacin and Levofloxacin are the most commonly prescribed fluoroquinolone drugs used to treat patients with MDR-TB. Despite these analogues displaying enhanced antimycobacterial activity *in vitro* and *in vivo*, levofloxacin was shown to be more cost-effective, and therefore more accessible in resource-limited high burden settings [18]. In comparison to moxifloxacin, gatifloxacin and levofloxacin, DC-159a, a relatively new fluoroquinolone analogue was shown to exhibit enhanced bactericidal activity against MDR-TB both *in vitro* and *in vivo* and may therefore be a promising new therapeutic candidate for reducing treatment time for both MDR- and drug-sensitive (DS)-TB [22, 23].

3.3 Clofazimine and TBI-166

Clofazimine is an antibiotic belonging to the class of Riminophenazines that is currently approved for the treatment of leprosy [19, 24]. Clofazimine possesses both antimicrobial and anti-inflammatory properties and although its mechanism of action is still unclear, the outer membrane of bacteria appears to be the primary target of this inhibitor [19]. Although Clofazimine has shown good activity against MDR- and XDR-TB, its efficacy in humans is still under investigation specifically concerning long term use and its major adverse effect of causing skin discoloration [25]. Clofazimine is mainly utilised in combination with other drugs in the second-line treatment of drug-resistant TB and has been classified as a Group 5 medicine by the WHO [24]. TBI-166 a new generation analogue of Clofazimine was demonstrated to exhibit superior antimycobacterial activity in comparison to its predecessor as well as reduced skin discoloration and is currently in a Phase 1 clinical trial [25, 26].

3.4 Sanfetrinem (Trinem beta-lactam)

Sanfetrinem cilexetil is an orally available tricyclic beta-lactam developed by Glaxo Smith Kline (GSK) in the early 1990's with broad antibacterial activity on both Gram-negative and Gram-positive bacteria. The development of this drug was halted after phase 2 clinical trials. However, it has recently been identified as a potential beta-lactam against *M. tuberculosis*, with an MIC of 1.5 μg/mL against H37Rv and an intracellular MIC of 0.5 μg/mL in THP1 monocytes. Furthermore, it has been reported that the drug showed potent activity against a range of susceptible and resistant clinical isolates with an MIC90 of 1–4 μg/mL. In an *in vivo* investigation, sanfetrinem cilexetil was comparable to meropenem and amoxicillin/clavulanate [27]. Similar to other carbapenems, it targets the cell wall by inhibiting the formation of peptidoglycan [28]. This drug is currently under pre-clinical investigation with a planned phase 1 clinical trial.

3.5 Spectinamide 1810 (Spectinamide)

Spectinamides are semisynthetic derivatives of spectinomycin with a narrow spectrum activity against *M. tuberculosis* and present its activity through selective inhibition of the bacterial S16 ribosomal subunit. One factor that contributes to their potent antitubercular activity is the evasion of efflux through the Rv1258c efflux pump. This feature makes spectinamides promising candidates against MDR TB, which have been shown to upregulate efflux pumps [29]. Two derivatives, 1599 and 1810 were investigated for their combinational effect in an infected mice model co-currently administering different combinations of the derivatives with Bdq, Emb, Inh, levofloxacin, linezolid, moxifloxacin, PA-824, Pza, and Rif. The researchers showed that spectinamide 1599 showed synergistic activity in combination with rifampicin and pyrazinamide [30]. Spectinamide 1810 is currently in pre-clinical investigation and being developed by Microbiotix, Inc.

3.6 Meropenem, Faropenem (Carbapenem Beta-lactam)

Meropenem is a carbapenem-type beta-lactam antibiotic which has shown bactericidal activity against susceptible and resistant *M. tuberculosis* strains. In combination with clavulanate, it was able to sterilise cultures within 14 days [31]. Meropenem is used in the treatment of a variety of bacterial infections. One phase

2 clinical trial on newly diagnosed TB has been completed, and an additional two trials are currently recruiting suitable candidates.

3.7 Thioridazine (phenothiazine)

Thioridazine was a drug used in the treatment of anxiety disorders and schizophrenia. Manufactured by Novartis, it was removed from the market in 2005 due to associated cardiac arrhythmias and other adverse effects. The removal of this drug had a devastating effect on patients being treated for schizophrenia, and a study in Finland indicated a doubling of hospital admitted relapsed patients after the withdrawal of the drug [32]. Thioridazine was coincidentally the only drug that appeared in the network analysis on the topic of repurposing drugs for TB (**Figures 2** and **3**). It has shown *in vitro* bactericidal activity against susceptible and resistant strains of *M. tuberculosis* as well as intracellular activity on human macrophages with limited cellular toxicity [33, 34]. A retrospective study on a trial conducted in Argentina on 17 XDR-TB patients revealed the potential use of this drug in a last-resort treatment. Thioridazine was combined with linezolid and moxifloxacin. Although clinically relevant adverse effects (neurotoxicity and haematological disorders) were observed, and two patients had to have the treatment halted, the combination was able to achieve negative cultures in 15 patients and status of "cured" in 11 patients. The authors have recommended the use of this combination for compassionate use [35].

4. Repurposed drugs in discovery

Numerous ongoing projects are in pre-clinical development across the globe, with collaborative research groups spanning across both industry and academia. Many of these groups form part of the Tuberculosis Drug Accelerator (TBDA) program. Selected repurposed drugs that are currently in pre-clinical development, and which have been assessed *in vitro* or *in vivo* will be discussed further. It is worth noting that several computational screening programs of approved drugs are also ongoing against known targets in *M. tuberculosis.*

4.1 Carprofen and Oxyphenbutazone

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a class of drugs that are generally used to relieve pain and reduce inflammation, mainly functioning by inhibiting the activity of cyclooxygenase enzymes involved in the regulation of inflammation and blood clotting [19]. In mouse models of TB, the common NSAIDs namely aspirin and ibuprofen were shown to decrease both the size and number of lung lesions and bacillary load as well as improve survival rates [36]. Studies have revealed that analogues in this family namely Carprofen and Oxyphenbutazone were found to exhibit bactericidal activity against mycobacteria through inhibition of mycobacterial drug efflux mechanisms and biofilm growth [19, 36]. Both their antimicrobial and anti-inflammatory properties combined with their low likelihood of adverse effects following administration make them very strong candidates for repurposing as TB treatment.

4.2 Disulfiram

Disulfiram is a nontoxic drug belonging to the family of Carbamates. It is primarily used to treat chronic alcohol addiction, but has demonstrated potent antimycobacterial activity against clinical isolates, MDR and XDR strains [19, 37].

Moreover, it was demonstrated that the bactericidal activity of Disulfiram is synergistically enhanced in the presence of the metal ion copper, with the mechanism of action of this compound still under investigation [37].

4.3 Metformin (Biguanides)

Metformin, a biguanide drug approved for glycaemic control in patients suffering from Type II diabetes mellitus, falls within the group of host-directed therapies against TB. Multiple adjunctive activities have been investigated. *In vitro* studies have shown a potentiation of the standard TB drugs, an increased immune response and mediation of phagosome-lysosome fusion. The phagolysosome fusion leading to the inhibition of bacterial growth is due to the expression of AMP-activated protein kinase, which in turn increases the production of mitochondrial reactive oxygen species (mROS) [38, 39]. The adjunctive properties and potential in TB treatment have been captured in two reviews [40, 41]. A phase II clinical trial investigating the safety and tolerability of metformin in TB/HIV patients is yet to start, and the investigation is planned to be completed in 2024.

4.4 Metronidazole (Nitroimidazole)

Metronidazole is a broad-spectrum antibiotic used in the treatment of gastrointestinal infections. Some parasitic infections including amebiasis, giardiasis and trichomoniasis are also treated by this drug [42]. The exact mechanism of this drug has not been fully elucidated, but it has been hypothesised that the drug renders its action through the blocking of nucleic acid synthesis via an intermediate of metronidazole and through the production of a toxic metabolite in anaerobic bacteria through the reduction of the nitro group by the redox potential of the electron transport chain [43]. It has been shown that metronidazole was able to inhibit the growth of mycobacterial bacilli under anaerobic non-replicating conditions but showed no activity under aerobic conditions [44]. *In vivo* studies in macaques (a non-human primate model), showed similar efficacy of inhibiting reactivation of latent TB, as compared to a combination of isoniazid and rifampicin [45]. In a phase 2 clinical trial investigating the effect of metronidazole vs. placebo on pulmonary MDR-TB, some efficacy was observed in sputum smears after 1 month of treatment, but the benefit was not sustained past 2 months of treatment. The study was ultimately halted due to the occurrence of peripheral neuropathies within the test subject group [46]. Although metronidazole is associated with several adverse effects, other and newer nitroimidazoles are extremely important within the clinical pipeline against TB. These include pretomanid and delamanid which are both part of multiple phase 2 and 3 clinical trials.

4.5 Tolcapone, Entacapone (catechol-O-methyltransferase (COMT) inhibitor)

Entacapone and tolcapone are two catechol-O-methyltransferase inhibitors used as an adjunct in the treatment of Parkinson's disease. Both have shown some activity against *M. tuberculosis* with a relatively high minimum inhibitory concentration (MIC) of 260 μM observed for entacapone, which was significantly lower than the cytotoxic concentration [47]. Their proposed mechanism against TB is what makes these molecules an interesting class to investigate. The mechanism is similar to isoniazid; however, they do not need enzymatic activation to bind to the enoyl–acyl carrier protein reductase (InhA) target. Furthermore, it has been proposed that it might be a possible treatment in MDR-TB, as it could evade the KatG activation associated with isoniazid resistance in many resistant strains [19, 47].

5. Target-based repurposing

An additional benefit of drug repurposing is the potential to identify or validate vulnerable targets and/or pathways that can be exploited for further drug development [8–10]. Bortezomib is the first human proteasome inhibitor approved for the treatment of multiple myeloma and mantle cell lymphoma [48]. Using a target mechanism-based whole-cell screen, bortezomib was identified as an inhibitor of the mycobacterial caseinolytic protease (ClpP1P2), with growth inhibitory activity, thus validating it as a druggable target [49]. Further investigations have focused on structural modifications of bortezomib to increase selectivity for the mycobacterial ClpP1P2 complex over the human proteasome while maintaining antimycobacterial activity [49–51]. The *M. tuberculosis* DosRST two-component regulatory system is important for survival under non-replicating conditions which is thought to contribute to the required prolonged therapy for TB, and is therefore considered a promising target for drug development [52]. Artemisinin is used for the treatment of Malaria and was identified as an inhibitor of *M. tuberculosis* DosRST during a whole-cell phenotypic high throughput screen and is currently in the hit-to-lead phase of drug development [52, 53]. In addition to the identification of promising repurposed drugs by whole-cell screening, recent efforts have focused on computational modelling and virtual screening of known drugs against targets of interest. Using this approach two drugs were identified as inhibitors of *M. tuberculosis* DNA gyrase (GyrB): echinacoside which has been investigated for the treatment of Parkinsons and Alzheimers, and epirubicin which is a treatment for breast cancer [54–56]. Virtual screening has also identified Sulfadoxine, Pyrimethamine, Lifitegrast and Silfenadil as inhibitors of *M. tuberculosis* MurB or MurE, enzymes involved in peptidoglycan synthesis [57].

6. Conclusion and future prospects

The need for novel treatment strategies for TB is becoming more urgent if the goal of a TB-free world is to be realised. While the current treatment regimens have a success rate of 85% for DS-TB, there is, unfortunately, an increase in the incidence of DR-TB, which only has a treatment success rate of 57% and harsh side-effects for patients [1]. The drug discovery pipeline is relatively rich with new material; however, the conventional screening and development strategies have led to the identification of multiple chemical scaffolds that inhibit the same targets, referred to as promiscuous targets e.g. DprE1, MmpL3 and QcrB [58]. Furthermore, the global economic climate has significantly reduced the available funding for scientific research and due to the low return on investment, several pharmaceutical companies no longer support in-house drug discovery programs for infectious diseases [6], further hampering the quest for new drugs with novel targets. To this end, drug repurposing provides an appealing strategy with several advantages as outlined above. The success of Rif, Linezolid and the fluoroquinolones provides strong support for drug repurposing for the treatment of TB. The high number of repurposed drugs in the discovery phase of compound development and in advanced clinical trials suggests that this strategy is becoming more widely accepted in the TB research community and has good potential for success. Furthermore, with the continual advances in computational biology and open sharing of compound data across disease areas, it is not unreasonable to expect a boost in drug repurposing research in the future. This could possibly further reduce the time and cost to develop repurposed TB drugs, and aid in trying to meet the global goals of eradicating TB.

Acknowledgements

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Chapter 6

Breast Cancer Drug Repurposing a Tool for a Challenging Disease

Jonaid Ahmad Malik, Rafia Jan, Sakeel Ahmed and Sirajudheen Anwar

Abstract

Drug repurposing is one of the best strategy for drug discovery. There are several examples where drug repurposing has revolutionized the drug development process, such as metformin developed for diabetes and is now employed in polycystic ovarian syndrome. Drug repurposing against breast cancer is currently a hot topic to look upon. With the continued rise in breast cancer cases, there is a dire need for new therapies that can tackle it in a better way. There is a rise of resistance to current therapies, so drug repurposing might produce some lead candidates that may be promising to treat breast cancer. We will highlight the breast cancer molecular targets, currently available drugs, problems with current therapy, and some examples that might be promising to treat it.

Keywords: drug repurposing, breast cancer, mechanism, non-oncology drugs, resistance

1. Introduction

Drug discovery is a multifaceted process that aims at identifying a therapeutic agent that can be useful in treating and managing certain medical conditions. This process includes identification of candidates, characterization, validation, optimization, screening, and assays for therapeutic effectiveness. If a molecule achieves acceptable results in these studies, then the molecule has to go through drug development processes and be recruited to clinical trials [1]. Several drug candidates (about 90%) have collapsed in early clinical trials due to unexpected results such as adverse effects or inadequate effectiveness [2, 3]. Drug development is probably among the most complicated and challenging processes in biomedical research. Apart from the already enormous complexities underlying pharmacological drug designs, additional significant challenges arise from clinical, regulatory, intellectual property, and commercial constraints. Such as challenging atmosphere has made the drug development process very sluggish and unpredictable [4]. The process of discovering and developing a new drug is a lengthy and expensive process taking somewhere from 10 to 15 years and costs about US$2–3 billion [1]. Despite massive sums of money being spent on drug development, no substantial rise in the new therapeutic drug agents in a clinical setting has been observed over several decades. Although overall global R&D spending for drug discovery has risen 10-fold from 1975 (the US $4 billion) to 2009 ($40 billion), the number of novel molecular entities (NMEs) approved has stayed essentially constant since 1975 (26 new drugs approved in 1976 and 27 new drugs approved in 2013) [5].

The essential step in discovering new drugs involves the evaluation of the safety and effectiveness of new drug candidates in human subjects, and it consists of four phases. In Phase I clinical trial, the candidate drug's safety is assessed in a small population (20–80 individuals) to establish safe dose range and uncover adverse effects. Phase II involves the examination of intervention for its effectiveness and safety in large populations (a few hundred people). Phase III further involves the assessment of drug efficacy in a large population (several thousand) and compares new drug candidates with standard or experimental treatments. Phase IV is conducted when the intervention is marketed. This study aims to track how well the approved treatment is performing in the general population and gather data on side effects that may arise from broad usage over time. Phase III studies determine whether or not a medication is effective, and if so, FDA clearance is granted. The FDA approves one anticancer treatment out of every 5000–10,000 applicants, and just 5% of oncology medicines entering Phase I clinical trials are approved in the end. Because of the increased cost and time frame for new medication development in recent years, patients with severe illness may die until alternative therapies are available if they develop resistance to current therapy [6]. In searching for an alternative treatment option for managing various diseases, including cancer, the researchers have shifted their focus to drug repurposing strategies.

The drug purposing or drug reprofiling or drug redesigning process explores the therapeutic use of existing clinically approved, off patent drugs with known targets for another indication to minimize the cost of therapy, time, and risk [7]. The huge benefit of drug repurposing is that the efficacy, pharmacokinetics, pharmacodynamics, and toxicity characteristics have previously been explored in preclinical and Phase I investigation. These drug moieties may thus be quickly made to proceed to Phase II and Phase III clinical trials, and hence related developmental costs might be substantially lowered [6, 8]. The failure risk in drug development is low because *in vitro* screening, *in vivo* screening, toxicity profile, chemical optimization, and formulation development have already been accomplished. Therefore, drug repurposing has made the pharmaceutical industry a desirable choice for investors. So the pharmaceutical companies and researchers have begun to make significant investments in drug repurposing, which offers a tremendous benefit over *de novo* drug design and development [9]. Therefore this new approach of drug purposing has reduced the timeline and cost of the drug development, notably in the case of FDA-approved repurposed pharmaceuticals, which will undergo faster clinical

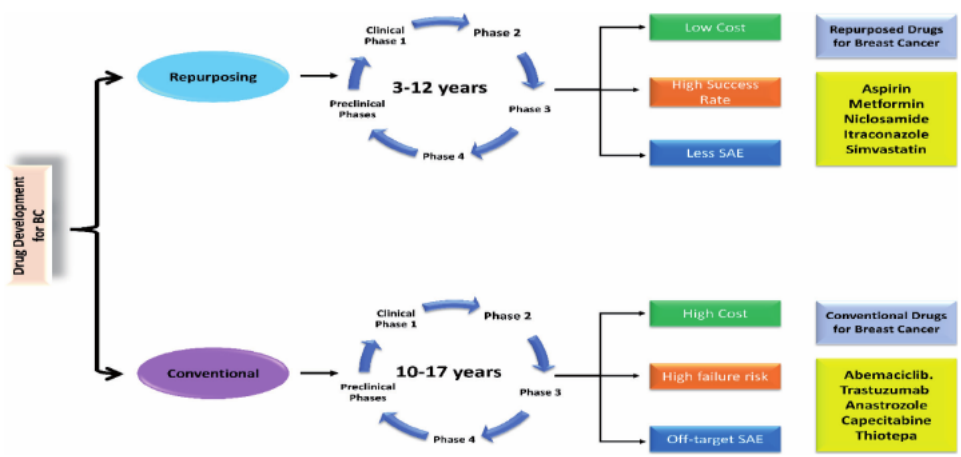

Figure 1.
Outline of developing new drug versus repurposing.

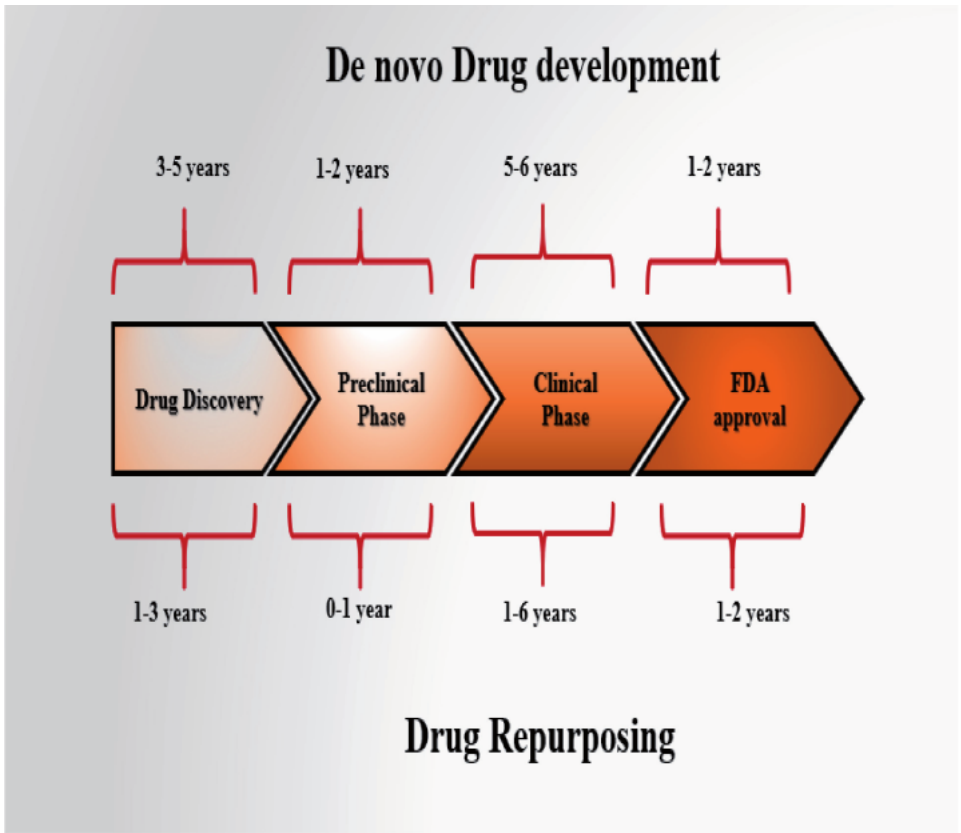

Figure 2.
The time taken by the conventional of process of drug development with respect to drug repurposing. Conventional drug development process takes around 5 years and the same can be minimized to 5 years.

trials because of already well-known safety and toxicological profile [10]. Outline of developing new drug verses repurposing is represented in **Figure 1**.

The development of new drugs for breast cancer like any other cancer is a multistep process that includes drug designing, synthesis, characterization, safety and efficacy assessment, and finally, regulatory approval (**Figure 2**). The overall process is very lengthy and involves significant financial expenditure [11]. Further, the sky-high cost of the therapies and associated side effects make it desirable to look for other approaches to manage cancer effectively. Therefore, concurrently with the synthesis and design of new therapeutic modalities, various strategies should be considered for repurposing various already approved drugs that may target this deadly disease.

2. Non-oncology drugs repurposed for breast cancer (preclinical data)

2.1 Aspirin

Aspirin was originally discovered in 1897 and was first commercialized as an analgesic. It has been utilized as an anti-inflammatory medication and for managing arterial and venous thrombosis [12]. Recent research has sparked interest in the usage of Aspirin for the prevention of various cancers. There are compelling evidences authenticating that regular use of low doses of aspirin results in a significant reduction in the occurrences and mortality of various cancers [13–17]. The

possibility that Aspirin has an anticancer benefit has received considerable interest nowadays, with a lot of research being done to figure out how successful it is in the prevention of colorectal cancer [18], lung cancer [19], gastric cancer [20], prostate cancer [21], and many other cancers including breast cancer. Because of the effect of Aspirin in several biological processes such as inhibitory effect on angiogenesis [22], cancer cell metastasis [23], causing cell apoptosis [24], etc., it is reasonable to predict that Aspirin will be beneficial when employed as an additional alternative treatment option for cancer patients. Aspirin directly inhibits the activity of the enzyme cyclooxygenase (COX-2) and thereby impedes the synthesis of prostaglandin E2 (PGE-2), which leads to cancer cell death [25]. Recent research also suggests that Aspirin may mediate anticancer potential through COX-independent pathways such as inhibition of NFκB [26], downregulation of survivin [20], targeting AMPK-mTOR signaling [27], Wnt signaling cascade [28], etc.

A study was conducted by Dai et al. reported that Aspirin possesses antiangiogenic and anti-metastatic potential in MDA MB 23 cell line by directly binding to the enzyme heparinase. The results were further confirmed *in vivo* experimentation [23]. Heparinase is an endo-β-D glucuronidase that is specific to heparin sulfate. It dissolves heparin sulfate chains of proteoglycans on the cell surface and extracellular matrix (ECM) that consequently contributes to the degradation of the extracellular matrix that further assists tumor invasion and metastasis [29]. Further, heparin also facilitated the release of angiogenic factor, vascular endothelial growth factor (VEGF) blocked by aspirin-mediated heparin inhibition [23]. Breast cancer cell lines (MDA MB 231 and MCF-7) showed a dose-dependent inhibitory effect on growth after treatment with Aspirin. The Aspirin further restricts the migration of these cells by preventing epithelial to mesenchymal transition through suppression of various mesenchymal markers such as vimentin and increasing expression of various epithelial markers such as Keratin-19 and E-cadherin.

Further inhibitory effect of TGF-β/SMAD4 signaling, as evident from decreasing the production of SMAD proteins, also contributes to the anti-metastatic potential of Aspirin [30]. In another study, Choi et al. demonstrated the effect of Aspirin in the MCF-7 cell line. It was observed that Aspirin alters the complex formation between Bcl-2 and FKBP38 and leads to the nuclear translocation of Bcl-2 and phosphorylation that causes its activation, contributing to its inhibitory effect on MCF-7 cell proliferation and also triggers apoptosis in cell lines [31]. In combination with exemestane, Aspirin showed synergy in inhibition of cell proliferation. Significant arrest in the G0/G1 phase was observed along with a more detrimental effect on COX-1 and Bcl-2 expression than individual therapy [32]. In addition, when combined with tamoxifen (which is used as a drug of choice for the estrogen receptor positive BC), it downregulates the level of cyclinD1. Subsequently, it arrests the cell cycle in phase G0/G1. In the same study, authors also reported that Aspirin inhibits the ER + ve BC cells growth and overcomes the resistance to tamoxifen in MCF-7/TAM cell line. Study demonstrated a new way to treat ER + ve BC in combination therapy of Aspirin and tamoxifen [33].

2.2 Metformin

Metformin (1,1-dimethyl biguanide hydrochloride) is a well-recognized biguanide derivative and has a long history of usage in managing type 2 diabetes (T2D). Because of the outstanding ability to lower plasma glucose levels, metformin has become the primary drug for managing T2D [34]. The drug was firstly approved in 1958 in the United Kingdom, and this decade-old drug is in the WHO's list of essential medicines [35]. Metformin belongs to the category of successful repurposed drugs and advanced into the clinical trials Phase 3/4 for its use in the

prostate, oral, breast, pancreatic, and endometrial cancers [6]. Various preclinical and clinical examinations have demonstrated the effectiveness of metformin in the treatment of various malignancies such as pancreatic cancer [36], gastric cancer [37], blood cancer [38], etc. A meta-analysis study on diabetic patients with breast cancer concluded that patients who were treated with metformin and neoadjuvant therapy had a higher pathological complete response rate (24%) compared with patients not undergoing metformin treatment (8%) [39]. Another meta-analysis study demonstrated 65% survival improvement when compared with control [40]. Metformin has increased the survival opportunity in type 2 diabetic patients suffering from invasive breast cancer [41]. Study also suggested that patients on metformin demonstrate improved in the survival and response to treatment [40]. The metformin uptake is mediated by the OCT1 in BC cells [42], which is reported to play important role in the BC cells as an anticancer activity [43]. Upon entry into the cells, it leads to increase apoptosis, anti-proliferative, anti-angiogenic, which seems to be mediated by the mTOR, Akt/MAPK pathway [44]. Study conducted by Shi et al., established that metformin can also inhibit the expression of the COX-2, suggested the potential of metformin in combination with others COX-2 inhibitor [45]. Low cost and stability of metformin make it a good candidate for the treatment of cancers when compared with available treatment options [46].

2.3 Itraconazole (ITC)

Itraconazole, a triazole antifungal drug, is a well-tolerable agent that is extremely effective against a wide range of fungal infections. Itraconazole is a highly potent and effective antifungal agent due to its active metabolite, hydroxyitraconazole, which also has significant antifungal action [47]. Itraconazole blocks ergosterol synthesis in the fungal cell membrane by inhibiting the enzyme 14α-demethylase and suppressing their growth [48]. It has emerged as a potent anticancer agent because of its ability to overcome chemoresistance prompted by P glycoproteins, altering various signaling pathways such as hedgehog (Hh) signaling cascade, Wnt/β-catenin pathway in cancer cells, and also preventing angiogenesis and lymphangiogenesis [49]. Itraconazole has been shown to have the ability to eliminate cancer cells by disrupting Hh signaling [50]. In invertebrates, the Hh signaling cascade is responsible for the regulation of complicated developmental processes. However, aberrant activation of this pathway plays a crucial role in carcinogenesis and cancer maintenance and contributes to chemoresistance, thus, targeting this pathway offers the potential therapeutic possibility [51]. Itraconazole was able to exhibit cytotoxicity in breast cancer cell lines by influencing mitochondrial membrane potential through induction of apoptosis, decreasing expression of Bcl-2, and enhancing the caspase activity. Itraconazole also promoted autophagic cell death via elevation of LC3-II expression, degradation of P62/SQSTM1, formation of autophagosomes. Hedgehog signaling is an important regulator of apoptosis and autophagy. Hence, inhibition of this signaling by Itraconazole results in cytotoxicity, tumor shrinkage, apoptosis, and autophagy in breast cancer both in *in vitro* and *in vivo* investigations [50, 52]. Anticancer activity is also reported in esophageal cancer, mediated by downregulating the HERK/AKT pathway [53]. A pilot study with 13 participants demonstrated that increased levels of Itraconazole in plasma were associated with the increased level of thrombospondin-1, angiogenesis inhibitor.

Additionally, the level of other growth factors such as fibroblast growth factor (FGF) and placenta-derived growth factor also decreased without any direct association with the Itraconazole [54]. When administered in combination with other cytotoxic agents, Itraconazole increased the response rate [55]. Researchers

are trying various ways to enhance the anti-neoplastic activity of itraconazole. One such example is the development of the modified lipid nanoparticles having Miltefosine (subtherapeutic dose), called M-ITC-LNC (Membrane additive itraconazole with lipid nanoparticles (Miltefosine). The results from the cytotoxicity studies demonstrated that the anticancer activity and selectivity significantly increased in MCF-7 BC cells compared with the ITC-solution and ITC-LNC without modification [56]. In another study, itraconazole was co-delivered with the doxorubicin by liposome (coated with the Pluronic P123), resulting in the increased anti-neoplastic activity in BC [57]. The combination of the verapamil and ITC with 5-FU decreased cell survival and proliferation.

Moreover, ITC and 5-FU are more effective in the treatment of BC [58]. Administration of the Itraconazole with erlotinib (tyrosine kinase inhibitor) increased the AUC and C_{max} by 10.8 and 2.78-fold, respectively, without any SAE [59]. Abovementioned all the studies reveal the potential of Itraconazole alone or in combination with other anticancer agents to treat BC.

2.4 Simvastatin

Simvastatin belongs to the class of statins and is a well-explored hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor that reduces cholesterol biosynthesis initially used to reduce cholesterol biosynthesis marketed in 1988 [60]. Clinical data suggest that statins are effective in BC management. Statins amplify tumor cell death and radiosensitivity in various cell lines, inhibit invasion and proliferation, and show anti-metastatic activity. Clinical trials conducted on breast cancer (inflammatory and TNBC) patients also favored these observations by representing improved mortality benefits for patients on statins [61, 62]. In the same context, Simvastatin is the most explored statin to explore the role of statins in cancer. Simvastatin targets the transcription factor NFκB that reduces the expression level of anti-apoptotic protein Bcl-xL, concomitantly inhibits the expression of anti-proliferative and proapoptotic tumor suppressor PTEN and hence inhibiting the growth of breast cancer cells. The elevation of PTEN expression results in the suppression of Akt phosphorylation. Akt activity is upregulated in many cancers by increasing cancer cell survival, inhibiting apoptosis, and increasing proliferation. Therefore, Simvastatin substantially decreased Akt phosphorylation concurrently with the reduction in expression of anti-apoptotic protein by dysregulation of NFκB, thus showing the anticancer activity against BC [63]. On administration of Simvastatin, the expression of PTTG1 (pituitary tumor-transforming gene 1) was also reduced in a dose-dependent manner in the MDA-MB-231 cell line. PTTG1 is the important gene involved in the invasion and metastasis of BC [64]. In the same cell line (MDA-MB-231), Simvastatin leads to fragmentation of the cell's nuclei, subsequently inducing apoptosis. It also enhanced the level of ROS in a dose-dependent manner, which causes oxidative stress and further DNA damage [65]. Apoptotic effects were due to the increased expression of miR-140-5p in a dose-dependent manner mediated by the activating transcription factor NRF1 [65]. Apart from the MDA-MB-231 cell line, Simvastatin effects were also explored in other breast cancer cell lines such as T47D, BT-549, and MCF-7, showing apoptotic inducer anti-proliferative activity [66, 67]. In *in vivo* studies with DMBA (dimethyl-Benz(a)anthracene) induced breast cancer rat model, Simvastatin reduced the tumor volume by around 80% [68]. Karimi et al. also explored its activity in breast cancer mice model and reported improved mortality and tumor volume compared with control [69]. Although Simvastatin's lipophilic nature makes it a good candidate for the BC treatment, the researcher tried to develop nano formulations to improve the delivery in a targeted specific manner and reduce the non-target side

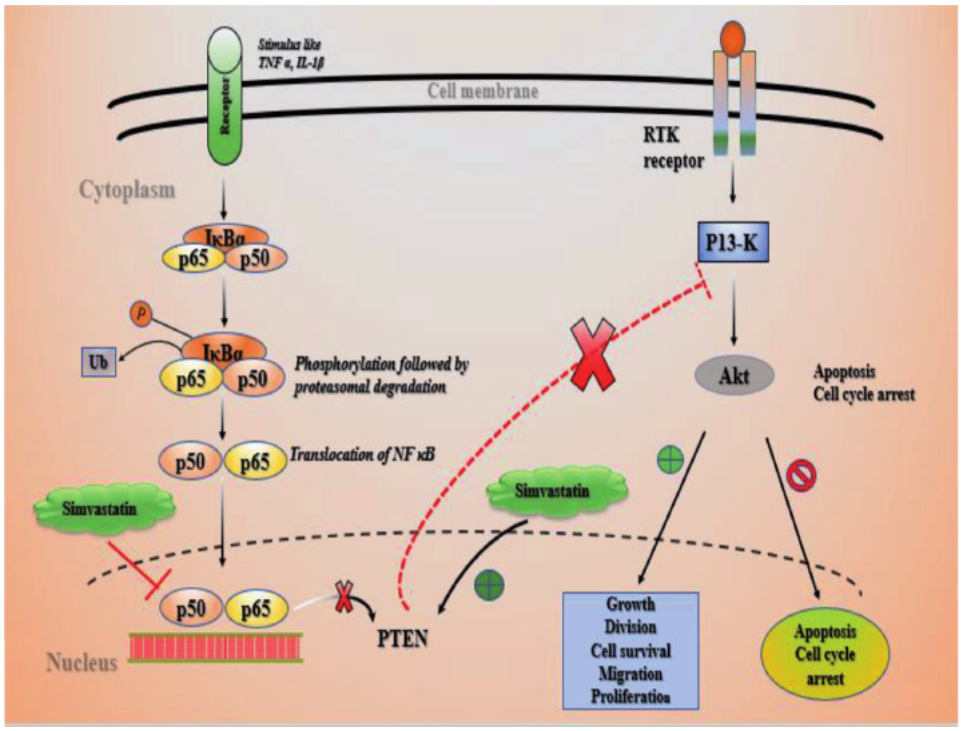

Figure 3.
Simvastatin acts via blocking p50–65 leading to activation of PTEN, which inhibits PI3K-Akt axis leading to inhibition of cell growth, division, survival, migration, and proliferation.

effects. Detailed mechanism of cell growth inhibition, division, survival, migration, and proliferation by Simvastatin is presented in **Figure 3**.

In the same series, Sed et al. used nanoparticles made of superparamagnetic iron oxide to Simvastatin delivery with enhanced anticancer activity in the PC-3 cell line. This action is mediated by inducing apoptosis and cell cycle arrest in the G2 phase [70]. Researchers from another lab developed poly D, L-lactide-co-glycolide (PLGA) with cholic-acid-based nanoparticles for Simvastatin release in a sustained and controlled manner for breast adenocarcinoma treatment. These nanoparticles showed maintainable and more efficiently inhibit tumor growth than normal Simvastatin [71]. Other formulations such as nanocapsule [72], nanoemulsions [73], liposomes [74], and immunoliposome [75] for Simvastatin were developed with increased anticancer activity in breast cancer cells. In a randomized placebo-controlled study, Simvastatin shows a better anticancer profile with the carboplatin and vinorelbine in metastatic breast cancer [76]. Consistency in the results from both clinical and preclinical studies suggests the vast potential of Simvastatin in treating breast cancer either alone or in combination. Moreover, the development of nanoformulations also provided advantages such as enhanced cytotoxicity, lower side effects, targeted delivery over the conventional available treatment options for BC.

2.5 Niclosamide

Niclosamide, an FDA-approved anthelminthic drug used to manage tapeworm infection, has been used almost from the last half of the century and included in the WHO's list of essential medicines. Recent research suggests that niclosamide has a wide range of therapeutic uses other than treating parasitic infection. Niclosamide's

Drug	Experimental model	Mechanism of action	Observation	Original indication	References
Aspirin	B16F10, MDA-MB-231, MDA-MB-435 xenograft mode MCF-7, MDA-MB-231 MCF-7, MDA-MB231	Inhibition of heparinase Inhibition of TGF-β/SMAD4 signaling pathway ↓EMT Apoptosis	↓Metastasis, ↓angiogenesis ↓Mesenchymal markers (vimentin, Snail, TWIST)	NSIAD	[23, 30, 31]
Itraconazole	MCF-7 and SKBR-3 breast cancer cell	Antiproliferative effect via inhibition of hedgehog signaling cascade ↑Apoptosis ↑Autophagy ↑Cell cycle arrest	↓Tumor size ↑Caspase 3 ↓Bcl-2	Antifungal drug	[50]
Niclosamide		↓EM T ↑Apoptosis Inhibition of stat signaling	↓Snail ↓Vimentin ↓Tumor growth ↑Caspase 3, ↓Bcl-2, ↓surviving, ↓Mcl-1 expression	Anthelminthic drug	[80, 81]
Simvastatin	MDA-MB-231, T47D, BT-549 and MCF-7 (*in vitro*) DMBA model (*in vivo*)	↓PTTG1, ↓Bcl-xL ↑ROS ↑miR-140-5p Inhibition of Akt and DNA damage	Anti-proliferative, induce apoptosis, and increased survival	Anti-hypercholesterolemic drug	[63–67]
Metformin	MDA-MB-231, MCF-7	Via mTOR, Akt/MAPK pathway COX-2 inhibition	Apoptosis, anti-proliferative, anti-angiogenic	Anti-diabetic drug	[44, 45]

Table 1.
Summary of the repurposed drugs for BC discussed in the chapter.

clinical application diseases include type 2 diabetes, endometriosis, neuropathic pain, bacterial and viral infections, including cancer [77]. The anticancer benefits of niclosamide have been shown in many malignancies such as colon cancer, lung cancer, prostate cancer in humans, as well as breast cancer by suppressing various cancer related pathways such as Wnt Notch, mTOR, STAT, and NFκB [78, 79]. The combinational treatment of niclosamide with cisplatin overcomes the resistance to cisplatin and induces an inhibitory effect on proliferation *in vitro* and reduced tumor size *in vivo*.

Further, niclosamide prevented the epithelial-mesenchymal transition (EMT) by suppressing mesenchymal markers such as snail and vimentin. The inhibitory effect on EMT and prevention of stem-like phenotype of TNBC by Niclosamide operate by disabling various abnormal signaling pathways such as Akt, ERK, and Src [80]. The niclosamide acts as a potent inhibitor of STAT signaling by preventing cancer cell proliferation, invasion, and metastasis by decreasing the phosphorylation of STAT3 that otherwise was found in 35% of breast cancer tissues. Furthermore, STAT3 promotes the expression of several key downstream genes involved in proliferation, cell survival, and angiogenesis in breast cancer [81]. Human monocyte cells were reduced to HUVECs in the presence of niclosamide. Niclosamide also inhibited VCAM-1 and ICAM1 protein expression in HUVECs. Niclosamide decreased HUVEC proliferation, migration, and development of cord-like structures. *In vivo*, niclosamide inhibits VEGF-mediated angiogenesis [77]. Niclosamide inhibited Wnt/Frizzled 1 signaling, mediated by the increased degradation of the Wnt co-receptor LRP-6 (low-density lipoprotein receptor-related protein 6) [82–84]. Osada et al. determined that on the administration of niclosamide, there was a decrease in Dvl2 expression, which further impeded the downstream signaling (β-catenin) [85]. Londoño-Joshi et al. reported that niclosamide administration also reduced levels of LRP6 and β-catenin in breast cancers [86]. In combination with doxorubicin, niclosamide induces apoptosis and synergistically increases breast cancer cell death. This action is mediated by Wnt/β-catenin pathway downregulation and arrest of the cell cycle by Niclosamide in G0/G1 while both doxorubicin and niclosamide increased ROS production, thus showing cytotoxicity [87, 88]. Niclosamide also showed synergistic anticancer activity with 8-quinolinol [89]. When niclosamide is administered with cisplatin, it could inhibit the invasion and cell stemness of breast cancer cells, mediated by downregulation of anti-apoptotic protein Bcl2 [90]. In a recently published study, albumin-bound niclosamide (nab-Niclo) (Albumin-based nanoparticle transport systems) was found to inhibit cell growth, induce cell death, mitochondrial dysfunction, and increase oxidative stress with DNA damage. This nab-Nicolo was appeared more effective than normal Niclosamide for BC treatment [91]. Taken together, all the data suggest that niclosamide alone and in combination with other drugs could be used for the normal BC and resistance BC all repurposed drugs for BC discussed in this chapter summarized in **Table 1**.

3. Conclusion

Drug discovery is a multifaceted process that aims at identifying a therapeutic agent that can be useful in treating and managing various ailments. This process includes identification of candidates, characterization, validation, optimization, screening, and assays for therapeutic effectiveness. As the mortality due to cancer is progressively increasing, we need effective therapy to treat breast cancer patients or improve survival. When any pharmaceutical organization starts developing a novel chemical entity for the BC, its cost and attrition rate are very high. Drugs

repurposing is how we can minimize the cost and attrition rate by using the already marketed drugs for a new use. Drug repurposing against breast cancer is one of the best alternatives to treat progressive ailments. In the above discussion, we have discussed various drugs that can be repurposed against breast cancer. It will be a game-changing scenario in the treatment of breast cancer. Certain challenges need to be rectified. However, there is a need for optimization of models and more screening of drugs at preclinical stages.

4. Future prospective

To tackle all the challenges associated with the drug development process for breast cancer, scientists need to shift their interest to the alternative drug development, that is, drug repurposing. All the BC repurposed drugs discussed in the book chapter show impressive results that suggest exploring more new non-cancerous drugs for cancerous use [92]. Using the drugs repurposing approaches alone and in combination with other drugs will also reduce the side effects associated with high doses. It will also reduce the cost of the drug development process, ultimately patient compliances and burden. Patients who could not afford the treatment due to the high cost can take treatment and improve survival. As the safety is already studied of drugs that seem a novel interest in the repurposing for BC, the chances of failure at the clinical level will also be less. With the advancement in drug repurposing, there is still a need to develop a valuable model of different types of cancers that mimic cancer. The development of such a model provides the actual clue for drug repurposing. So far, the advantages we discussed, there are some challenges associated with the drugs repurposing such as patent issue, regulatory consideration, inequitable prescription that need to be overcome so, more and more pharma companies show their interest in drug repurposing. It is expected that drug repurposing will achieve the milestone that is currently not possible with the conventional available treatment for cancers in the future. Furthermore, new nanoformulations need to be developed for the targeted and specific delivery of repurposed anticancer drug to avoid the off-target side effects.

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The Future Perspectives of Drug Repurposing and Treatment for the Drug Resistant Breast Cancer: A Review

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Abstract

Breast cancer is a major health concern as it is the second leading cause of death from cancer. There are several well-known risk factors that contribute to breast cancer. Despite the various treatment options available, complete cure is still difficult due to heterogenicity of BC subtypes. As a result, identifying BC subtypes is critical for determining the optimal treatment approach. Over the last several years, new drugs targeting particular therapeutic targets have resulted in significant advances in the treatment of breast cancer. Nonetheless, resistance to treatment is the "major" issue, and a significant increase in survival rates has been the main focus for researchers. The purpose of this review article is to provide a broad overview of the molecular basis of drug resistance in breast cancer, as well as a detailed assessment of current treatment options, potential new treatment methods for drug-resistant breast cancer and repurposed drugs used for treatment. The possibility of non-cancer drugs being studied for breast cancer in the future, as well as the obstacles and bottlenecks of drug repurposing, is also highlighted. Finally, we go through present problems and future prospects in drug-resistant breast cancer therapy.

Keywords: Breast Cancer, Endocrine Resistance, Oestrogen Receptor Modulation, Drug Repurposing

1. Introduction

Breast cancer is the most frequent disease among women, according to the World Health Organisation (WHO), and it is the second leading cause of death from cancer, after lung cancer. It is considered a severe health concern that affects patients' quality of life as well as their psychological well-being, It is the main cause of death among women aged 45 to 55 years old. The incidence of breast cancer is expected to grow by 85 per 100.000 women by 2021 [1]. Experts estimate that by 2050, there will be approximately 3.2 million new BC cases each year worldwide,

based on population increase. Although there is no single risk factor for the majority of breast cancer patients, there are a number of well-known risk factors, including obesity, lack of physical activity, consumption of alcohol, hormone replacement therapy, increased breast density, and inherited genetic susceptibility due to mutations in autosomal dominant genes, which contribute for 5–10% of all breast cancer cases in the United States [2]. Treatment for BC is difficult since it is a heterogeneous illness with various subtypes that have different but distinct clinical and biochemical characteristics. As a result, identifying BC subtypes is critical for determining the optimal treatment approach [3]. Breast cancer may be in situ or invasive, with in situ tumours being the easiest to cure. Invasive breast cancers, especially invasive ductal carcinoma (which accounts for 80% of all invasive breast cancers), are a major source of concern. While receptor-specific therapy is used to treat the first two types of breast cancer, chemotherapy remains the mainstay of TNBC treatment [4]. BC is characterised as basal-like or non-basal-like according on the cell type of origin (luminal or basal/myoepithelial cell compartment). The aforementioned, also referred to as "triple-negative," contributes approximately 10% of all BCs. Understanding the etiological heterogeneity of BC subgroups will aid in directing therapy, predicting survival, and impacting preventive measures due to the complexity of biology [5]. With the standardisation of systemic chemotherapy as the gold-standard method for most cancer types and the moderate increase in both survival rates and toxicity reduction, targeted therapy has undoubtedly garnered the greatest scholarly attention and financing from the pharmaceutical sector. Nonetheless, resistance to treatment is the "major" issue, and a significant increase in survival rates is still a pipe dream for researchers. It is important to note that tremendous progress has been achieved in the area of breast cancer research during the last decade. The 'battle' against this mysterious and aggressive form of cancer, however, is still ongoing [6]. The purpose of this review article is to provide a broad overview of the molecular basis of drug resistance in breast cancer, as well as a detailed assessment of current treatment options and potential new treatment methods for drug-resistant breast cancer. Finally, we go through present problems and future prospects in drug-resistant breast cancer therapy.

2. Breast cancer risk factors

BC is associated with the following epidemiological risk factors: (a) a younger age at the first menstrual cycle and during the first birth, (b) pre-menopause is the prime factor in most BC patients, (c) civilization is an unavoidable outcome of increased risk for BC fatalities, (d) socio - economic background is an unbiased predictor of sophisticated extent at assessment in breast cancers, and (e) obesity and higher BMI are epidemiological risk factors for BC (**Figure 1**).

3. Pathogenesis

Breast cancers typically begin as ductal hyperproliferation and progress to benign tumours or even metastatic carcinomas as a result of continuous stimulation by carcinogenic agents. Breast cancer initiation and progression are influenced by tumour microenvironments such as stromal effects and macrophages. When only the stroma, not the extracellular matrix or the epithelium, was exposed to carcinogens, the mammary gland of rats may be driven to neoplasms. Macrophages may create a mutagenic inflammatory microenvironment, allowing cancer cells to avoid immune rejection and increase angiogenesis. The normal and tumour-associated

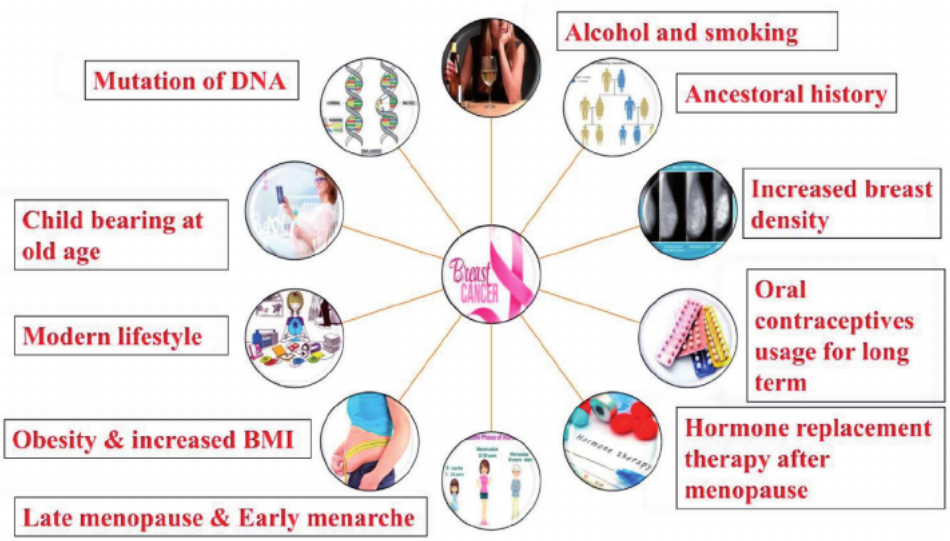

Figure 1.
The risk factors of breast cancer.

microenvironments exhibit different DNA methylation patterns, suggesting that epigenetic changes in the tumour tissue may promote tumorigenesis. Cancer stem cells (CSCs), a new type of malignant cell seen in tumours, have been linked to tumour genesis, migration, and relapse. This minor group of cells can auto renew and is resistant to chemotherapy and radiation. They may be produced from stem

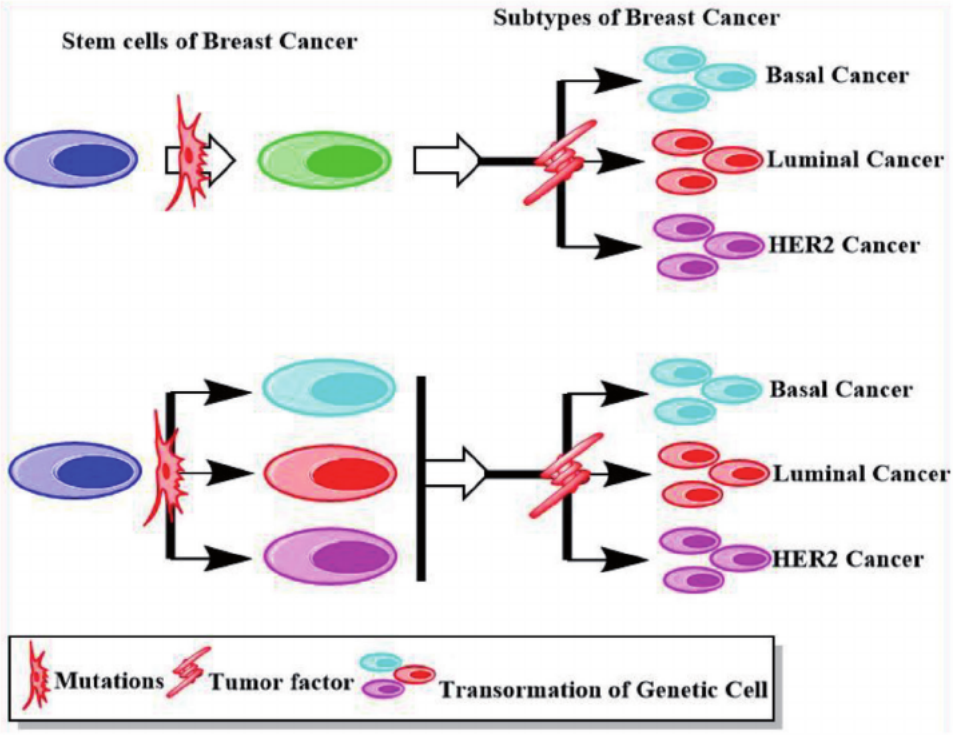

Figure 2.
The possible hypothesis for onset and development of breast cancer.

cells or progenitor cells in normal tissues. Ai Hajj was the first to identify breast cancer stem cells (bCSCs), demonstrating that as few as 100 bCSCs can create new tumours in infected mice. Luminal epithelial progenitors are more likely than basal stem cells to give rise to bCSCs. Wnt, Notch, Hedgehog, p53, PI3K, and HIF are all signalling pathways involved in the auto-renewal, multiplication, and migration of bCSCs. However, more research is needed to fully comprehend bCSCs and create ingenious ways for their eradication. The cancer stem cell theory and the stochastic theory are two distinct hypotheses for breast cancer initiation and progression. All tumour subtypes, according to the cancer stem cell theory, are derived from the same stem cells or transit-amplifying cells (progenitor cells). Various tumour features will result from acquired genetic and epigenetic alterations in stem cells or progenitor cells (**Figure 2**). According to the stochastic theory, each tumour subtype begins from a single type of cell (stem cell, progenitor cell, or differentiated cell) (**Figure 2**). Any breast cell can acquire random mutations over time, eventually transforming it into a tumour cell if enough mutations are accumulated. Despite the fact that both theories are backed up by evidence, neither can adequately explain the origins of human breast cancer [7].

4. Types of breast cancer

According to a review, breast cancer is divided into invasive and noninvasive breast cancers **Figure 3.**

4.1 Non-invasive breast cancer

It's a malignancy that has not spread beyond the lobule or ducts in which it's found [8]. Ductal carcinoma in situ is an example of a kind of non-invasive breast cancer. Ductal carcinoma in situ develops when abnormal cells form inside the milk ducts but do not spread to nearby tissue or to the outside. The term "in situ" means "in place." Atypical cells may develop and mature into invasive breast cancer even if they have not spread beyond the lobules or ducts.

4.2 Lobular carcinoma in situ (LCIS)

Breast lobules form as a result of this kind of breast cancer. Outside of the lobules, the breast cancer has not spread into the breast tissue. Non-invasive breast cancer is typically diagnosed as lobular carcinoma in situ.

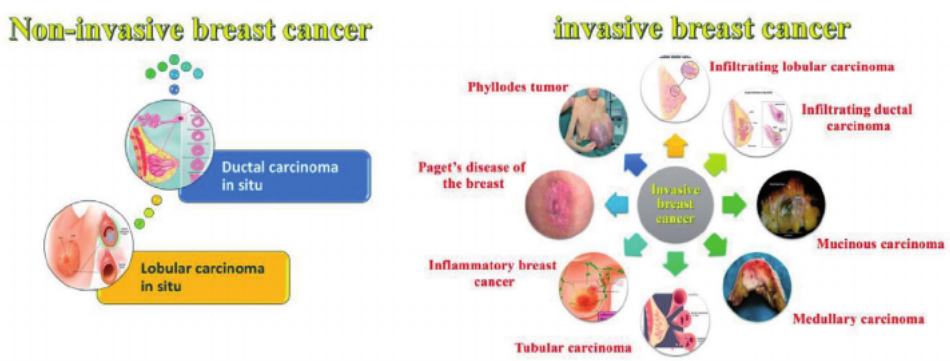

Figure 3.
Types of breast cancer.

4.3 Ductal carcinoma in situ

It is the most common type of non-invasive breast cancer, because it only affects the breast duct. Ductal comedocarcinoma is an example of ductal carcinoma *in situ*.

4.4 Invasive breast cancer

When abnormal cells from the lobules or milk ducts break off and come into contact with breast tissue, this condition occurs. Through the immune system or the systemic circulation, cancer cells may spread from the breast to other areas of the body. They may migrate early in the formation of the tumour, when it is small, or later, when it is large. Invasive breast cancer is the most common kind of cancer in women. Metastatic breast cancer is defined as invasive breast cancer that has spread to other parts of the body. The brain, bones, lungs, and liver are the most frequent organs to which these cells travel. These cells separate and grow irregularly once again, resulting in new tumours. Although new forming cells are appearing in many parts of the body,but still remains to be breast cancer cells [9, 10].

4.5 Infiltrating lobular carcinoma (ILC)

Invasive lobular carcinoma is another name for infiltrating lobular carcinoma. ILC begins in the breast milk glands (lobules), but it may spread to other parts of the body.

4.6 Infiltrating ductal carcinoma

Invasive ductal carcinoma is also known as infiltrating ductal carcinoma. IDC begins in the breast milk ducts and spreads to the duct wall, infecting the fatty tissues of the breast and perhaps other areas of the body.

4.7 Medullary carcinoma

Invasive breast cancer with a distinct normal and medullary tissue border is known as medullary carcinoma.

4.8 Mucinous carcinoma

Mutinous carcinoma, sometimes called colloid carcinoma, is an uncommon kind of breast cancer characterised by cancer cells that produce mucus. Females who have mutinous carcinoma have a better prognosis than those who have other kinds of invasive carcinoma.

4.9 Tubular carcinoma

Tubular invasive breast carcinomas are a form of invasive breast carcinoma. Tubular carcinoma had a better prognosis than other forms of invasive carcinoma.

4.10 Inflammatory breast cancer

Inflammatory breast cancer causes swollen (red and heated) breasts with bulges and/or broad ridges, which happens when cancer cells block lymph arteries or channels in the skin surrounding the breast. Inflammatory breast cancer is an uncommon kind of cancer that rapidly spreads. Throughout treatment,

all multidisciplinary techniques, including as radiation therapy, surgery, chemotherapy, and imaging, must be carefully integrated. Since the first publication on this subject, neoadjuvant chemotherapy has resulted in a substantial increase in overall survival and has taken the place of locoregional treatments like radiation and surgery, resulting in long-term improvements in this disease [11, 12].

4.11 Paget's disease of the breast

It's an uncommon kind of breast cancer that produces visible changes to the breast's nipple. Red itchy rashes around the nipple, which may occasionally spread to the rest of the body, are among the symptoms. Paget's disease of the breast differs from other skin problems like eczema and psoriasis in that the other skin problems usually affect both breasts and can start at the areola rather than the nipple of the breast, whereas Paget's disease of the breast usually affects only one breast and starts at the nipple of the breast rather than the areola. Men and women are equally affected by Paget's disease, which contributes for 1–3% of all breast malignancies.

4.12 Phyllodes tumour

Tumours caused by Phyllodes may be benign or malignant. Phyllodes tumours grow in the breast's connective tissues and may be surgically removed. Phylloides tumours are very rare; in the United States, less than 10 women die of this kind of breast cancer each year [13–15].

4.13 Triple-negative breast cancer

Breast cancer is now well understood to be a diverse disease with several subforms characterised by their distinct clinico-pathological features, prognosis, and treatment responses. The absence of progesterone receptor, human epidermal growth factor receptor 2, and oestrogen receptor expression characterises triple-negative breast cancer. This kind is primarily destructive, and it is more frequent in premenopausal females. It accounts for 10–15 percent of cases in white females, with a higher frequency.

5. Stages of breast cancer

5.1 Stage 0

This is a non-invasive tumour stage in which both cancerous and non-cancerous cells are enclosed within the boundaries of the breast part where the tumour begins to grow, with no evidence of their invasion into the surrounding tissues of that part; ductal cell carcinoma in situ (DCIS) is an example of this tumour stage [16].

5.2 Stage 1

Invasive breast cancer is described as this stage, and microscopic invasion is conceivable. It is divided into two stages: 1A and 1B. Te category 1A refers to a tumour that is up to 2 cm in diameter and does not include any lymph nodes, while stage 1B refers to a tiny collection of cancer cells bigger than 0.2 mm discovered in a lymph node [17].

5.3 Stage 2

Stage 2 is divided into two categories: 2A and 2B. The tumour is detected in axillary lymph nodes or sentinel lymph nodes in Stage 2A, but there is no tumour in the breast. The tumour may be 2 cm in diameter or 5 cm in diameter. Stage 2B, on the other hand, defines a tumour that is bigger than 5 cm in diameter but does not reach the axillary lymph nodes [18].

5.4 Stage 3

It's broken down into four sections: 3A, 3B, and 3C. Stage 3A refers to a tumour that has caused swelling or ulceration on the breast skin and has spread to up to 9 axillary lymph nodes or sentinel lymph nodes, whereas stage 3B refers to a tumour of any size that has caused swelling or ulceration on the breast skin and has spread to up to 9 axillary lymph nodes or sentinel lymph nodes. Because it has progressed to 9 axillary lymph nodes or sentinel lymph nodes, stage 3B breast cancer is deemed inflammatory. Tumour spread to 10 or more axillary lymph nodes, as well as lymph nodes above and below the clavicle, is classified as stage 3C [19].

5.5 Stage 4

This is the late and metastatic stage of cancer, in which the disease has spread to other internal organs including the lungs, bones, liver, and brain **Figure 4** [20].

6. Clinical breast cancer diagnosis techniques

The assessment methods and popular imaging techniques that will aid physicians in providing better care to patients and advancing clinical diagnosis are discussed below.

1. History and physical examination of breast cancer

   The clinical history of breast cancer patients is used to assess the risk of developing cancer and to show the existence or absence of breast disease symptoms [21]. Age at menarche, menopausal status, prior pregnancies, and usage of hormone replacement therapy or oral contraceptives beyond menopause are all factors to consider. Personal as well as family history should be carefully investigated. Breast soreness, weight loss, bone pain, tiredness, and nipple discharge are just a few of the symptoms to check into. During a physical examination, doctors look at the breasts, the area around the neck and collarbone, and the armpits (axillae). Any anomalies in the breasts, such as lumps or other breast cancer signs, are investigated. Lymph nodes, which are often enlarged in breast cancer patients, are also assessed [22, 23].

2. Self examination

   The value of breast self-examination is debatable since no benefit in terms of decreased mortality has been demonstrated. Most doctors teach women to do monthly self-examinations in order to get familiar with their normal structure and to give them authority over their own healthcare. Self-examination may reveal irregularities in breast size and form. Sreedharan et al. performed research at hospitals in the United Arab Emirates. A self-administered structured questionnaire was utilised to look at self-examination and knowledge practises. This research [24] produced satisfactory outcomes. These studies have

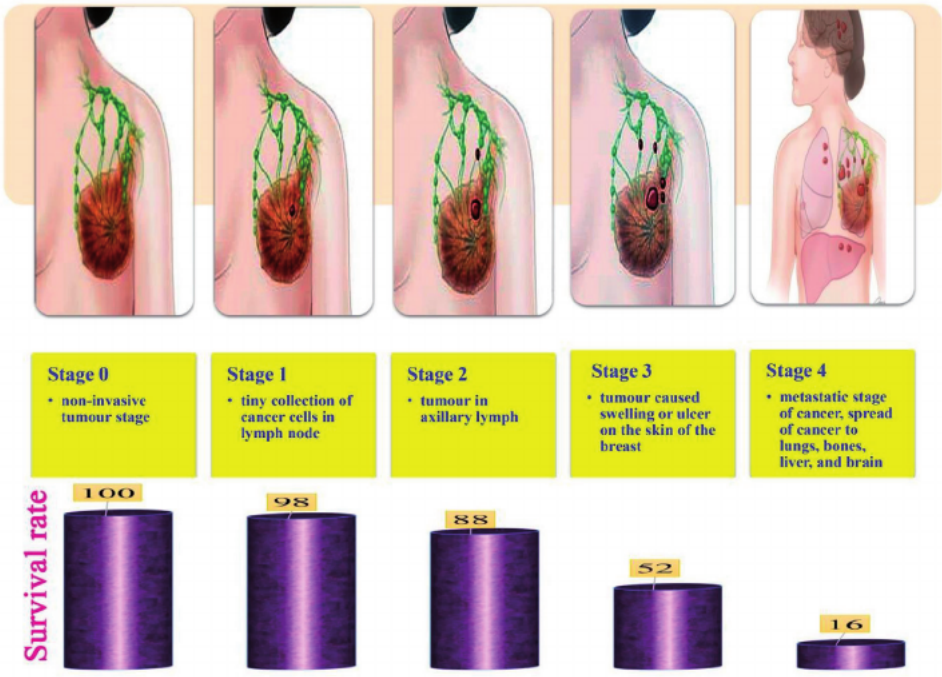

Figure 4.
Stages in development of breast cancer.

demonstrated that a continuous breast cancer education programme may help people become more aware of the disease. Ceber et al. performed research on Turkish women's breast self-examination and health attitudes, concluding that early detection of breast cancer may avoid physical diseases and early mortality. He further claimed that just one out of every seven patients with breast cancer receives a timely diagnosis [25].

3. Mammography

Mammography (MG) is the recommended method for screening and detecting breast cancer, and it aids physicians in gathering clinic data on BC patients. The data indicates that early MG screening may decrease the death rate of BC patients by 30 percent to 40 percent [26]. Meanwhile, only 4 percent –10 percent of BC patients have MG as a positive diagnostic finding. With the passage of time, MG continues to grow. The two primary methods for diagnosing BC patients in clinic are contrast-enhanced mammography (CEM) and digital breast tomosynthesis (DBT) [27, 28]. Age, ethnicity, personal history, radiologist expertise, and technique quality all influence mammography sensitivity. In high-density breasts in premenopausal women, sensitivity may be decreased. Mammography has a number of disadvantages, including the use of ionising radiation, inability to diagnose thick breasts, high false-positive and false-negative rates, and an unpleasant examination.

4. Ultrasonography

Breast ultrasonography is a low-cost and commonly available screening method that detects malignancies by rebounding acoustic waves off breast tissue. To identify the anatomy of the human breast, an ultrasonic transducer

is utilised to detect the acoustic waves reflected off it. Although less efficient than mammography, breast ultrasonography increases cancer detection rates in high-risk women and helps in the identification of cysts and solid masses. For women at high risk of breast cancer, pregnant women, and those who are unable to undergo mammography, breast ultrasonography has been recommended as a supplement to mammography. When breast ultrasonography and mammography are used together, the sensitivity of the imaging increases at the cost of specificity and biopsy rates. Because the reverberant characteristics of healthy and malignant tissues are so similar, breast ultrasonography fails to identify many tumours. It also necessitates the employment of qualified radiologists, which has a big impact on sensitivity and specificity [29].

5. Magnetic resonance imaging

MRI creates pictures at different cross-sections by mixing a strong magnetic field with RF signals. Breast MRI is suggested for high-risk women, but not for the general population because of its high rate of inaccuracy, higher expense, time commitment, insufficient number of units, requirement for trained radiologists, and lack of therapeutic effect. The American Cancer Society (ACS) has published recommendations for utilising MRI as a complement to mammography, and for specific demographic groups, such as BRCA mutation carriers and those at higher risk of complications, annual MRI scans are advised. In women at high risk of breast cancer, MRI is less specific but more sensitive than mammography and ultrasound in identifying small lesions [30].

6. Nuclear medicine

It is a kind of molecular imaging in which a person is administered a radioactive substance, and the radiation released by the radiopharmaceutical is shown by sensitive emission detectors such as gamma cameras and PET detectors located outside the patient's body. The combination of a CT scanner and a gamma camera, as well as a CT scanner and a PET scanner, is a significant advance in the identification and localization of disease.

7. Single photon emission computerised tomography (SPECT)

This method employs single photon radionuclides that produce gamma rays, such as gallium-67, iodine-131, and technicium-99. It's a fast and precise scan for the organ of concern. It may be used over the whole body, is quite safe in terms of radiation dose, and is effective in detecting both primary and metastatic tumours. The abbreviation PET/CT refers to positron emission tomography. PET/CT is also low-radiation since it utilises positron-emitting radionuclides including oxygen-15, fluoride-18, and carbon-11 to produce positrons. In positron emission tomography, a radioactive version of glucose, such as [18F] fuoro-2-deoxy-d-glucose, is a typical tracer. Tissues with greater metabolic needs, such as developing cancer cells, absorb the tracer more readily, which is seen on the scan. Using a combination of CT and PET, significant information about a range of situations impacting the different organs of the body may be readily mapped. PET/CT is extremely sensitive and accurate for predicting opaque and distinct areas of loco-regional lymph nodal extent and/or far-away metastases that are not apparent on conventional imaging, with up to 25% of patients having their staging changed. This technique is used to plan management by describing the primary disease's spread. It's also utilised in re-staging and treatment follow-up after a return of a managed disease [31].

8. Tumour markers

Tumour markers should be examined at all stages of breast cancer therapy, diagnosis, and screening, including metastasis prediction, treatment, and diagnosis, according to Porika et al. Thirteen distinct breast cancer tumour indications are investigated, six of which are new to the guideline. The different variations have been proven to be therapeutically useful and are recommended for use in clinical practice [32]. In order to avoid over- or under-interpreting the therapeutic potential of a few studies, the physician must be aware of the limits in the combined specificity and sensitivity of each sign. With these restrictions in mind, submitting tissue, germ-line, and soluble tumour markers for clinical trials may assist individuals who are at risk for or have breast cancer get back on track with their treatment.

9. Breast biopsy

Breast biopsies are the most effective way to find out whether you have breast cancer. Biopsies of the breast occur in a range of sizes and forms. To enhance diagnosis accuracy and remove as many false negative results as possible, breast imaging, breast self - examination, and biopsy are all performed at the same time (triple test).

a. Fine needle aspiration

A thin prickle is used to extract cells from an abnormal area or a breast nodule. Ultrasound may be used to guide the prickle. A local anaesthetic may be used to anaesthetize the region where the prickle will be inserted.

b. Core biopsy

A larger prickle is used to extract a core of tissue from the abnormal region or breast lump. It is usually performed under a limited anaesthesia, so the breast is unaffected, and the patient may feel no pain or discomfort depending on when the anaesthetic is administered. For the length of the core biopsy, an MRI, ultrasound, or mammography may be utilised to guide the procedure.

c. Vacuum-assisted stereotactic core biopsy

Different small tissue samples are obtained through a single tiny incision in the skin using a prickle and a suction-type device in this core biopsy. It is done with the use of a local anaesthetic. To guide the prickle into place, an MRI, ultrasound, or mammography may be used. During the procedure, the patient may feel a bit uneasy.

d. Surgical biopsy

A surgical biopsy is performed if the abnormal site is too small to be biopsied by another technique or if the biopsy result is unclear. A guide wire may be inserted into the breast prior to the biopsy to aid the medical practitioner in locating the abnormal tissue. A local anaesthetic may be administered, and the wire can be guided into place using MRI, ultrasound, or mammography. After that, a general anaesthesia is used to perform the biopsy. Along with the wire, a little region around the breast tissue and lump is removed.

7. Treatment methods

The goal of breast cancer treatment is to maintain quality of life while extending life expectancy. Breast cancer treatment methods vary based on the stage of the disease, its size, location, whether it has spread to other organs of the body, and the individual's physical state. Targeted treatments, hormone treatment, radiation therapy, and surgery are being used to treat breast cancer.

1. Surgery

This is the most common treatment option for people with breast cancer that has not spread to other parts of the body, and it's also a viable option for those with more advanced stages of the disease. The amount of tissue removed with the cancer varies according on the cancer's features, whether it has spread, and the patient's particular emotions. The following are a handful of the most common types of surgery:

a. Lumpectomy (breast conserving surgery)

According to the American Cancer Society [33], a lumpectomy, also known as a selective mastectomy, is a practice that requires removing the portion of the breast that contains the malignant tumour, as well as some healthy tissues and lymph nodes around it, while leaving the rest of the breast preserved as much as possible. This operation is often performed on women in the early stages of cancer, but in addition to the surgery, the patient will need additional treatments such as radiation, chemotherapy, or hormone replacement therapy. Most surgeons and patients, particularly if the woman is going to lose her breast, prefer a lumpectomy over a full breast removal at first. Tenderness, transient inflammation, sclerosis, and a change in the look of the breast are all possible side effects of a lumpectomy.

b. Mastectomy

The purpose of a mastectomy is to reduce the chance of developing breast cancer. Bilateral preventive mastectomy reduces the risk of getting breast cancer but does not fully remove it. Aromatase and tamoxifen are more effective than contra-lateral preventive mastectomy in reducing the risk of contra-lateral breast cancer. Mastectomy is the most efficient treatment for a disseminated instance of breast cancer in whom a lumpectomy was ineffective. Nonetheless, most women experience feelings of asexuality, loss of self-image, and melancholy as a result of breast loss [34].

c. Reconstructive surgery

Females who have had a mastectomy might consider having their breasts renovated, either immediately or later. It is used to improve the appearance of the breast after tumour surgery. All ladies who have had a mastectomy should be given the choice of reconstructive surgery [35]. Mastectomy is a very straightforward surgical procedure that usually requires 1–2 days in the hospital. Breast mass deficiency alters the patient's appearance and makes it difficult to wear certain types of clothes. The use of an external prosthesis to address these issues may be uncomfortable and abrasive, especially for women with large breasts. The most serious side effect after mastectomy is the psychological impact of the physical and cosmetic changes, which may include anxiety,

sorrow, and poor effects on body image and sexual activity [36]. Females with breast cancer who are unable to get breast-conserving therapy or who have a higher genetic risk of breast cancer often seek breast reconstruction. Breast reconstruction methods now available are varied and may include the use of a prosthetic implant, an autologous tissue flap, or both. Cancer may recur in the rebuilt breast regardless of the technique used; furthermore, in autologous tissue flaps repaired breasts, minor complexity such as fat necrosis may occur. Breast reconstruction, according to studies, restores body representation, demonstrates vitality, femaleness, and sexuality, and has a positive impact on the patient's emotions of comfort and life quality [37].

2. Ovarian ablation as adjuvant therapy for breast cancer

Breast cancer patients have been treated with ovarian ablation. Radiation-induced ovarian ablation, surgical removal of the ovaries, and long-term use of luteinizing hormone-releasing hormone (LHRH) analogues are all options for ovarian ablation. Furthermore, there are a few theories that cytotoxic chemotherapy may help premenopausal women with breast cancer by causing ovarian ablation. Many of the case studies and clinical trials of ovarian excision conducted in the past had methodological flaws. A meta-analysis of randomised clinical trials found that women who had ovarian ablation as an adjuvant therapy had a significant improvement in overall survival and disease-free survival compared to those who did not. According to a study of the literature, ovarian ablation may be used as an alternate treatment for breast cancer [38].

3. Breast cancer therapy by class

Various classes of therapeutic agents are employed for breast cancer treatment:

a. Alkylating agent: cyclophosphamide (nitrogen mustard)

b. Anti-metabolite: methotrexate (folic acid analogue), 5-fluorouracil &capacitabine (pyramidine analogues)

c. Natural product: vinorelbine (vinca alkaloid), paclitaxel (taxane), doxorubicin (antibiotic)

d. Hormone and antagonist: tamoxifen (anti oestrogen), letrozole&anastrazole (aromatase inhibitors)

e. Miscellaneous: trastuzumab (monoclonal antibody), lapatinib (Protein tyrosine kinase inhibitor)

4. Chemotherapy

Chemotherapy is the process of eliminating cancer cells with the help of specific medications. It may be administered both before and after surgery, depending on the patient's health. Docetaxel, Paclitaxel, Platinum agents (cisplatin, carboplatin), Vinorelbine (Navelbine), Capecitabine (Xeloda), Liposomal doxorubicin (Doxil), Cyclophosphamide (Cytoxan), Carboplatin (Paraplatin), and other drugs are included in chemotherapy, according to the American Cancer Society [39]. However, it has a number of negative side effects. The following are some of the most frequent breast cancer treatment regimens.

Cyclophosphamide is used to treat breast cancer metastases by preventing DNA replication and cell division. This prodrug is converted into active metabolites by hepatic intracellular enzymes (i.e. 4 hydroxy cyclophosphamide, aldophosphamide, acrolein and phosphor amide mustard). The medication has been utilised in the treatment of breast cancer as an adjuvant therapy in conjunction with CMF or an anthracycline.

Platinum compounds such as **Carboplatin and Cisplatin** are used to treat breast cancer as monotherapy or in conjunction with other cancer treatments. Platinum compounds have been investigated for their effect on DNA structure and stability, and a variety of platinum-DNA adducts have been discovered in vivo and in vitro. The impact of these different lesions on DNA replication, their potential to introduce mutations, and their susceptibility to DNA repair methods have all been measured in the early studies. Platinum (IV) compounds may cause further DNA damage, perhaps as a result of the cell's conversion to platinum (II) compounds. About 20–35 percent of patients with metastatic breast cancer who were receiving monotherapy responded to carboplatin treatment. The medicines Gemcitabine and Taxanes are often used in conjunction with Platinum compounds.

Capecitabine is a fluoropyrimidine oral prodrug that, when converted to 5-FU by the thymidine phosphorylase enzyme, has comparable effects as 5-FU infusion. It has been used in conjunction with taxanes to treat metastatic breast cancer that has progressed.

Gemcitabine (also known as difluorodeoxycytidine) is a pyrimidine nucleotide that inhibits RNA synthesis and DNA replication and is used to treat malignancies of the lung, bladder, and breast. Weekly IV injections of gemcitabine are well tolerated.

Vinorelbine binds to tubulin, causing mitotic metaphase to be disrupted. According to several studies, this medication has shown encouraging effects in advanced breast cancer.

Although metastatic or secondary breast cancer is difficult to cure, it may be managed for years. Chemotherapy may be used to control metastatic breast cancer and slow or stop its progression. It may also be used to reduce the severity of certain symptoms. Other treatments may be started before to or concurrently with chemotherapy.

5. Aromatase inhibitors

These are compounds that target aromatase, the enzyme complex that is responsible for the last step in the synthesis of oestrogen, in order to reduce oestrogen formation. Letrozole, exemastane, and anastrozole are examples of third-generation aromatase inhibitors that are currently used. A randomised clinical study that looked at the efficacy of these chemicals in treating women with advanced breast cancer found that they are quite beneficial. Females treated with aromatase inhibitors had a lower risk of developing contralateral breast cancer than women treated with tamoxifen, according to a clinical trial [40].

6. Anti–angiogenesis drugs

Antiangiogenic therapy for breast cancer has a lot of potential and several ongoing studies are attempting to better understand the optimal care settings and mediator selection. Research suggests a link between endocrine resistance and cancer dependency on angiogenic networks in patients with oestrogen receptor positive tumours, suggesting a possible therapeutic benefit in combining endocrine treatment with antiVEGF mediator. Results from randomised clinical trials highlight the wide range of responses to antiVEGF therapy, indicating that a better selection of patient subgroups is needed to maximise the benefits of these treatments. The identification

of biomarkers for treatment response is a single area of intense interest, however most studies to far have failed to find a correlation between cancer-associated indicators such as cancer mutations and EGF expression and scientific response.

7. Radiation therapy

Radiation treatment is beneficial in early breast cancer patients, according to Zhou et al. This research looked at 143 women who had breast conserving surgery and received either regular or intraoperative radiation treatment. There was substantial local control of the tumour after 54 months of follow-up. Radiation treatment uses high-energy beams to destroy cancer cells. Only the cells that are treated are affected by this treatment. After breast cancer surgery, radiation treatment may be used to eliminate any residual cells in the chest region [41].

a. Brachytherapy

It's a type of radiation treatment. Accelerated partial breast irradiation is a term that comes to mind. It just focuses radiation in the general region where the cancer was discovered. This might potentially eliminate the need for whole-breast radiotherapy. The number of management sessions is also reduced.

8. Protein tyrosine kinase inhibitor

Lapatinib is an orally active, reversible EGFR and HER2 tyrosine kinase inhibitor whose primary mechanism of action tends to be driven by HER2. When trastuzumab-treated HER2-positive breast cancer developed, lapatinib was authorised for use in combination with capecitabine; it's also utilised as a first-line treatment for HER2-positive metastatic breast cancer in combination with letrozole. Lapatinib and chemotherapy combined achieved a 22 percent response rate and a 27 percent clinical value rate in patients who had previously been treated with trastuzumab, and as prophylaxis, it achieved 12.4 percent to 25 percent clinical value rates; however, constrained resistance to lapatinib was observed in some cases [42, 43].

9. Gene therapy for carcinoma of the breast

Gene therapy is a kind of treatment that attempts to correct particular molecular defects related to breast cancer growth and progression. Cancer development is linked to mutated BRCA1 and p53 genes, which have been identified as cancer genetic markers. [44]. Cancer gene modification techniques may allow for selective targeting without presenting substantial hazards to non-cancer cells since cancer cells are the only ones that suffer mutational inactivation of gene activity in these circumstances. Even BRCA1 and p53 have been found to limit tumour cells without mutations in these genes, suggesting that so-called gene modification methods may be more effective than previously believed. These and other genes have been discovered as possible targets for gene substitution therapy as cancer genetics has become more well-known. Early patient investigations using BRCA1 and p53 gene therapy have shown a lot of encouraging indications of effectiveness, but they have also highlighted areas where additional clinical trials are required before these treatments may be widely utilised in breast cancer patients.

10. Cancer stem-cell therapy for breast cancer

The cancer stem-cell idea is based on recent breast biology studies. According to two key aspects of this theory, cancer arises in progenitor cells or mammary

stem cells as a result of a dysregulation of the normally tightly controlled mechanism of self-renewal. As a result, cancers contain a cellular component that retains basic stem-cell functions including self-renewal, differentiation, and tumorigenesis while also being accountable for cellular heterogeneity. Advances in the stem-cell field have assisted the identification of stem cells in both normal and malignant breast tissue. The finding of these stem cells has assisted in identifying the origins of human breast cancer's genetic complexity. In the early diagnosis, prevention, and treatment of human breast cancer, the cancer stem-cell hypothesis is critical. Dysregulation of stem cell renewal pathways is linked to both sporadic and hereditary breast cancers. These abnormal stem cells might be utilised to create novel cancer prevention methods. Moreover, because breast cancer stem cells may be resistant to chemotherapy and radiation, efficient targeting of this cell type may be required for the development of novel effective treatments for breast cancer.

11. Monoclonal antibodies

Trastuzumab is a physiologically active, humanised monoclonal antibody that acts against the extracellular domain IV of HER2 and has increased survival rates in HER2/neu positive breast cancer patients. This monoclonal antibody is clinically safe and effective when used in a three-week cycle, and it may also be used in conjunction with paclitaxel, gemcitabine, vinorelbine, or carboplatin.

12. Immunotherapy

To combat cancer cells, it makes use of the body's immune system. One of the examples is a cancer vaccination. Vaccines are made using cancer cell parts or cancer cells themselves. These cells activate the immune system, which aids in the attack and destruction of cancer cells. Immunotherapy has become an important component in the treatment of breast cancer. At the moment, HER2 targeted treatment is a significant element of HER2 over expressing breast tumour therapy.

Trastuzumab, in combination with the newer additions of pertuzumab and TDM1, provides significantly better breast cancer prediction.Immunotherapies are progressing in the field of development, with several FDA-approved antibody treatments being utilised in adjuvant and metastatic situations. Current gains in targeted treatments, robust specific immunotherapy, and grip ensure that general endurance in the adjuvant context will continue to improve. The very precise and focused vaccination treatment method not only avoids the side effects of contemporary standard of care medicines, such as active and passive immunotherapies like ipilimumab, but also provides a remedial strategy for those who are not HER2-overexpressing. Despite the fact that vaccinations for breast cancer have been mostly unsuccessful in previous clinical studies, the majority of these studies were done in the setting of advanced age metastatic disease, which is an unfavourable environment for medicines designed to halt, rather than manage disease. Immunogenicity is now showing a connection with medical response in adjuvant situations, according to current clinical research.

8. Drugs used for breast cancer

FDA approved and clinical status of investigational drugs for breast cancer treatment is listed in **Table 1** [45].

Anticancer agent	Target & application	Clinical status	Type
5-fluorouracil	Palliative treatment of breast cancer	Approved	Treatment
Abemaciclib	HR^+ and $HER2^-$ advanced/metastasized cancer	Approved	Treatment
Abemaciclib (LY2835219)	Rb^+ TNBC that is recurrent,locally advanced,metstatic or cannot be removed by surgery	Phase II	Treatment
Ado-Trastuzumab emtansine	$HER2^+$ metastasized and recurrent cancer has already been treated with trastuzumuab and a taxane	Approved	Treatment
Alisertib with or without fulvestrant	Locally advanced ot metastatic, endocrine – resistant breast cancer	Phase II	Treatment
Anastrozole	Postmenopausal women early stage, HR^+ metastatic breast cancer advanced breast cancer that has gotten worse after treatment with tamoxifen citrate	Approved	Treatment
Anastrozole or letrozole	HR^+ stage II-III breast cancer that can be removed by surgery	Phase I	Treatment
Cabozantinib with or without fulvestrant	HR^+ metastatic stage cancer with bone involvement	Pilot phase II	Treatment
Capecitabine	Metastasized cancer whose dieases has not gotten better with other chemotherapy *metastatic breast cancer	Approved *phase II	Treatment
Cyclophosphamide	Breast cancer *mesothelin-targeted T-cells after treating patients with metastatic, mesothelin expressing, $HER2^-$ breast cancer	Approved *phase I	Treatment
Dendritic cell vaccine + gemcitabine hydrochloride	Metastatic breast cancer	Pilot early phase I	Treatment
Docetaxel	Locally advanced or metastasized breast cancer that is node –positive and can be removed by surgery	Approved	Treatment
Docetaxel + carboplatin	Neoadjuvant treatment of stage II-III TNBC	Phase II	Treatment
Doxorubicin hydrochloride	Adjuvant therapy for braest cancer that has spread to the lymoh nodes after surgery	Approved	Treatment
Epirubicin hydrochloride	After whose cancer has spread to the lymph nodes under the arm	Approved	Treatment
Eribulin mesylate	Metastasized breast cancer who have already been treated with anthracycline and taxane *brain metastases from breast cancer	Approved *phase II	Treatment
Eribulin mesylate or paclitaxel	Recurrent stage IIIC-IV breast cancer	Randomised phase III	Treatment
Eribulin mesylate with or without pembrolizumab	HR^+ and $HER2^-$ stage IV breast cancer	Phase II	Treatment

Anticancer agent	Target & application	Clinical status	Type
Everolimus	Advanced $HR^+/HER2^-$ and has not gotten better after treatment with letrozole or anastrozole	Approved	Treatment
Exemestane	Early stage and ER^+; postmenopausal women who have already been treated with tamoxifen citrate –postmenopausal with stage 0-II ER^+ breast cancer before surgery	Approved *randomised phase IIb	Treatment
Fulvestrant	Postmenopausal women with HR^+ and $HER2^-$ advanced cancer	Approved	Treatment
Gemcitabine hydrochloride	Metastasized breast cancer that has gotten better with other chemotherapy	Approved	Treatment
Goserelin acetate	Premenopausal and perimenopausal women with advanced breast cancer	Approved	Treatment
Ixabepilone	Advanced metastasized who have not gotten better with other chemotherapy	Approved	Treatment
Lapatinib ditosylate	$HR^+/HER2^+$ breast cancer	Approved	Treatment
Letrozole	Early stage, HR^+ / $HER2^+$ advanced metastatic breast cancer	Approved	Treatment
Megestrol acetate	Palliative treatment of advanced disease in breast cancer	Approved	Treatment
Methotrexate	Breast cancer *breast cancer and leptomeningeal metastasis	Approved *phase II	Treatment
Neratinib	Stage IV $HER2^+$ breast cancer	Phase II	Treatment
Olaparib	Metastatic breast cancer with certain mutations in the BRCA1 or BRCA2 genes whose have $HER2^-$*triple negative non-metastatic breast cancer who have completed definitive local treatment and chemotherapy**metastatic breast cancer with DNA repair gene mutation	Approved *randomised Phase III**phase II	Treatment
Paclitaxel	Breast cancer	Approved	Treatment
Paclitaxel albumin-stabilised nanoparticle formulation (Abraxane)	Recurred (come back) or metastasized cancer	Approved	Treatment
Palbociclib (Ibrance)	HR^+ and $HER2^-$advanced or metastasized cancer	Approved	Treatment
Pamidronate sodium **Raloxifene hydrochloride**	• Invasive breast cancer in postmenopausal women who have osteoporosis • Selective benzothiopene oestrogen receptor modulator (SERUM) with lipid lowering effects and activity against osteoporosis • Oestrogen receptor	Approved	Treatment

Anticancer agent	Target & application	Clinical status	Type
Ribociclib	HR^+ / $HER2^-$ metastasized cancer who has not been treated with hormone therapy *ER^+ breast cancer	Approved *randomised phase II	Treatment
Tamoxifen citrate	Metastasized (spread to other parts of the body) breast cancer	Approved	Treatment
Thiotepa	Breast cancer	Approved	Treatment
Toremifene	Metastasized breast cancer and postmenopausal with ER^+ or ER^-	Approved	Treatment
Trastuzumab	Breast cancer that is $HER2^+$	Approved	Treatment
Trastuzumab emtansine	HER2 amplified or mutant advanced cancer	Phase II	Treatment
Vinblastine sulfate	Breast cancer that has not gotten better with other treatment	Approved	Treatment
Combination therapy **AC** **A = doxorubicin hydrochloride (Adriamycin)** **C = cyclophosphamide**	Primary, recurrent and metastatic breast cancer	Approved	Treatment
AC-T **A = Doxorubicin hydrochloride (Adriamycin)** **C = cyclophosphamide** **T = paclitaxel**	Adjuvant treatment of breast cancer	Approved	Treatment
CAF **C = cyclophosphamideA = Doxorubicin hydrochloride (adriamycin)** **F = fluorouracil**	Adjuvant treatment of nonmetastatic breast cancer alone for treatment of metastatic breast cancer	Approved	Treatment
CMF **C = cyclophosphamide** **M = methotrexate** **F = fluorouracil**	Adjuvant setting for the treatment of nonmetastatic breast cancer or alone for the treatment of metastatic breast cancer	Approved	Treatment

Anticancer agent	Target & application	Clinical status	Type
FEC **F = fluorouracil** **E = epirubicin hydrochloride** **C = cyclophosphamide**	Adjuvant setting and also for the treatment of recurrent and metastatic breast cancer	Approved	Treatment
TAC **T = docetaxel (Taxotere)** **A = Doxorubicin hydrochloride (adriamycin)** **C = cyclophosphamide**	Adjuvant treatment for breast cancer	Approved	Treatment

Table 1.
List of FDA approved and clinical status of investigational drugs for breast cancer treatment (source: NH-NCI, U.S).

9. Endocrine resistance for breast cancer

ER is expressed in around 70% of breast malignancies and plays an important role in their genesis and progression. Because of the involvement of ER in ER+ breast cancer, endocrine treatments such as aromatase inhibitors (AIs), selective oestrogen receptor modulators (SERMs), and selective oestrogen receptor degraders are commonly used to treat these tumours (SERDs). While hormone treatments have been successful in avoiding recurrence, about 20% of these tumours acquire resistance to hormone therapies and will return.

10. Drugs that block oestrogen receptors

These medicines operate by preventing oestrogen from driving the growth of breast cancer cells.

1. Selective oestrogen receptor modulators (SERMs)

The "selective" in the acronym SERMs alludes to the unique regulation of the oestrogen receptor and the downstream effect on ER signalling that happens inside various organs. Tamoxifen, for example, is known to have anti-proliferative (or antagonistic) effects in breast tissue while having agonistic or partial agonistic effects on the uterus, bone, and heart. In both the usage of SERMs and the creation of new medicines, the ratio of therapeutic benefit to negative tissue-specific effects has been an essential factor to address [46].

a. Tamoxifen

Tamoxifen has been effectively used to treat breast cancer in both premenopausal and postmenopausal women at all stages. It's utilised as a palliative treatment for those who have advanced cancer, as well as an adjuvant treatment after surgery for node-negative or positive cancer. Tamoxifen has consistently prolonged disease-free intervals as a postsurgical adjuvant therapy for early breast cancer with a low frequency of side effects. It is possible to achieve a 20% decrease in 5-year mortality, with the reduction being most noticeable in women over 50. Tamoxifen is used to reduce the risk of breast cancer and invasive breast cancer in women who are at high risk for the disease, as well as those who have ductal carcinoma in situ. Negative oestrogen receptor tumours do not respond to treatment [47].

b. Role of tamoxifen:

For individuals with oestrogen receptor (ER)-positive breast cancer, anti-oestrogen tamoxifen has been the endocrine therapy of choice. Tamoxifen decreases the risk of recurrence following surgery when used as an adjuvant treatment. Tamoxifen provides an objective clinical response in half of the individuals with recurrent illness. The cancer, on the other hand, will eventually become hormone-independent, meaning it will no longer respond to tamoxifen. Despite significant research, resistance mechanisms remain mostly understood [48].

Tamoxifen's hopeful profile spurred a slew of clinical studies and decades of anti-oestrogen research, which revealed new details about ER biology and its link to ER-dependent malignancies. There have been several randomised studies of adjuvant tamoxifen in early breast cancer patients. Before recurrence, information on

every woman in any randomised study of adjuvant tamoxifen versus no tamoxifen that began before 1990 was sought in 1995. The overall effects of tamoxifen proved to be minor among these women, therefore following studies of recurrence and total mortality are limited to the remaining women [49].

The effects of 1–2 years of tamoxifen and around 5 years of tamoxifen in the studies comparing tamoxifen vs. no adjuvant tamoxifen are summarised in Tamoxifen versus No Tamoxifen. The studies are separated by ER status, which is categorised as ER-poor, ERpositive, and ER-unknown, according to the recognised importance of the original tumour's hormone receptor status. Current and future assessments of receptor state may be more predictive of response as procedures for assessing receptor status advance. ER measures were, on average, extremely significant predictors of response to 5 years of adjuvant tamoxifen, despite the fact that it may be difficult to characterise the receptor assays employed in these studies many years ago. Many of the effects and side effects of tamoxifen in ER breast cancer patients are random [50].

2. Selective oestrogen receptor degraders (SERDs)

A selective oestrogen receptor degrader or downregulator (SERD) is a medication that binds to the oestrogen receptor (ER) and causes the ER to be degraded and therefore downregulated in the process. They're utilised with earlier types of medicines including selective oestrogen receptor modulators (SERMs) and aromatase inhibitors to treat oestrogen receptor-sensitive or progesterone receptor-sensitive breast cancer.

Selective oestrogen receptor degraders (SERDs) are oestrogen receptor antagonists that also cause proteasome-mediated ER degradation. Fulvestrant is a therapy for ER+ advanced breast cancer that has been authorised by the FDA [51].

a. Fulvestrant

Fulvestrant is an oestrogen receptor antagonist that inhibits and destroys oestrogen receptors. This medication is not a SERM; rather, it works as an anti-oestrogen throughout the body. It's referred to be an oestrogen receptor degrader that's selective (SERD). Fulvestrant is at least as effective and safe as comparator endocrine treatments in postmenopausal women with advanced hormone-sensitive breast cancer. Fulvestrant is a safe and effective systemic medication that can be regarded as a viable therapeutic option for postmenopausal women with hormone-sensitive advanced breast cancer in the treatment sequence [52].

Fulvestrant is a steroidal ER antagonist that was developed for its lack of agonism in almost all types of tissues studied, but it was subsequently shown to be a SERD that causes ER to be ubiquitinated and destroyed by the proteasome. It is, in fact, the only FDA-approved treatment for postmenopausal women who have relapsed on hormone therapy and have advanced ER-positive breast cancer. Fulvestrant, on the other hand, has an unfavourable pharmacokinetic profile and requires a painful intramuscular injection to be administered (500 mg dose). Stable-state plasma concentrations require 3–6 months to achieve, even with improved loading-dosage regimens (500 mg dose on days 1, 15, and 29). Its overall therapeutic efficacy is limited by the poor ER turnover seen in patient cases (less than 50%), compared to complete receptor downregulation shown in in-vivo breast cell line investigations. As a result, there is still an unmet medical need for a potent orally available SERD capable of reaching higher levels of malignant exposure [53].

b. Mechanism associated with ER Suppression:

Resistance to oestrogen suppression or inactivation of ER by other methods (SERMs/SERDs) is linked to and/or caused by mechanisms. Although the phrase "endocrine resistance" technically refers to resistance to oestrogen suppression, we use it here to refer to oestrogen or ER suppression resistance.

In ER+ metastatic breast cancer, endocrine resistance is an unavoidable outcome (MBC), As a result, when CDK4/6 inhibitors (e.g., palbociclib, ribociclib, abemaciclib) are added to antiestrogens, progression-free survival in patients with ER+ MBC is significantly increased compared to antiestrogens alone. The addition of CDK4/6 inhibitors to antiestrogens abrogates some of the resistance mechanisms. However, in early-stage cancers, they might still be important drivers of hormone resistance. The **Figure 5** was indicating that the activation of HER2, EGFR, FGFR, and Other RTKs Promotes Endocrine Resistance, RTK activation is augmented by PI3K and MAPK signalling, which induces ER phosphorylation and promotes ligand-independent ER activation (most often by mutation or amplification). NF1 loss-of-function mutations activate Ras in a constitutive manner, which can activate the PI3K and MAPK pathways as well. In a ligand-independent way, ER phosphorylation increases transcription of ER-regulated genes. ER and oncogenic RTK signalling both target CCND1, the gene that encodes cyclin D1. RTKs activate additional transcription factors

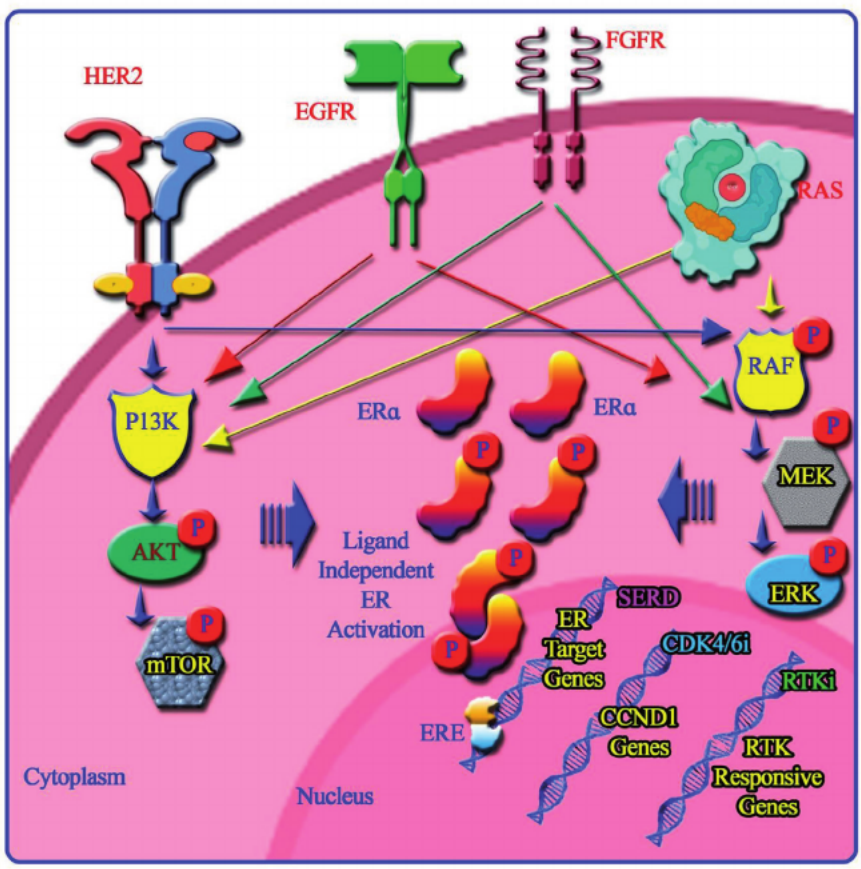

Figure 5.
Activation of HER2, EGFR, FGFR, and other RTKs.

that promote ER-independent survival in addition to ER. The combination of an ER antagonist with the appropriate RTK inhibitor CDK4/6 inhibitor might potentially overcome RTK-mediated endocrine resistance. Sensitivity to endocrine treatment and resistance to it in ER+ breast cancers are similar to other hormone-dependent malignancies including prostate cancer and endometrial cancer [54, 55].

11. Concept of selective oestrogen receptor modulation

SERMs are estrogenic and antiestrogenic molecules that have a wide range of effects. Two SERMs are now accessible in clinical trials: tamoxifen for breast cancer prevention and raloxifene for osteoporosis prevention. Tamoxifen was first created as an antiestrogen to treat breast cancer. Tamoxifen's widespread use as a therapy for all stages of ER-positive breast cancer in men and women has been assisted by its low risk of adverse effects. Concerns about the effects of an antiestrogen on bone density and the risk of CHD were raised when the strategy of testing long-term (5 years) tamoxifen therapy in ER-positive, lymph node-negative women and the proposed testing of tamoxifen as a preventive agent in high-risk women were proposed in the mid-1980s. Tamoxifen, on the other hand, is not a pure antiestrogen; it has antiestrogenic as well as estrogenic properties [56]. According to laboratory studies, tamoxifen is a selective oestrogen in areas like bone but an antiestrogen in breast tissue, preventing carcinogenesis and tumour development. Laboratory investigations dating back to the 1980s [57, 58] have confirmed raloxifene's SERM activity.

11.1 Mechanisms of action

Even though exact molecular mechanism of oestrogen or SERMs at the ER is unknown, two ERs govern oestrogen activity in target tissues: 1) ER, the traditional ER [59]; and 2) ER, which controls the action of ER and decreases tamoxifen's oestrogen-like effects [60, 61]. Although the crystal structure of the whole ER has yet to be determined by x-ray crystallography, data on the ligand-binding domains conjugated with estrogens and SERMs has been published. The outer forms of oestrogen and SERM complexes have been better understood as a result of this information. Oestrogen receptors (ERs) are nuclear transcription factors that bind estrogens, dimerize, and form a transcription complex with coactivators and other molecules to help unwind DNA. At oestrogen-responsive genes, RNA polymerase produces messenger RNA. SERM–ER complexes appear to alter the signal transduction route to oestrogen-responsive genes (through oestrogen response elements [EREs]) by binding a corepressor protein or activating fewer or different coactivators. This is, however, a simplistic model of oestrogen and antiestrogen action that overlooks the nuances of SERM function.

12. Drugs repurposed for breast cancer treatment

The commercially approved drugs that were originally used for diseases other than breast cancer are discussed in the following section. These medicines, on the other hand, are now being used or researched for breast cancer treatment. The drug candidates repurposed for breast cancer are divided into categories based on how they work (**Table 2**).

Drug	Chemical name	Mechanism	Original indication
Alkylating agent	Cyclophosphamide	Inhibits DNA replication by damaging genetic material of the cell	As immuno-modulator in autoimmune diseases
	Thiotepa		Immunosuppressant
Anthracyclins	Doxorubicin	DNA intercalation	Antibiotic from *Streptomyces peucetius* bacterium
	Capecitabine		Colon cancer
Antimetabolite	Fluorouracil	False building block incorporation during cell growth	Keratoacanthomas, actinic keratosis, and skin warts
	Gemcitabine		Anti-viral drug
	Methotrexate		Leukaemia
CDK 4/6 inhibitor	Palbociclib, Palbonix	Interferes with cell cycle	CDK 4/6 inhibitor
	Tamoxifen		Albright syndrome, ovulation induction
HT-SERM	Toremifene	Binds to ER	Infertility with an ovulatory disorders
	Raloxifene		Osteoporosis in postmenopausal women
HT-Aromatase inhibitor	Letrozole	Lowers oestrogen amount	Induction of ovulation
	Anastrazole		Induction of ovulation

Table 2.
A list of repositioned drugs approved for breast cancer treatment.

13. Executive summary

Breast cancer is still a significant public health problem, even though it was first reported more than 3500 years ago. This is particularly true in light of most societies' substantial and harmful lifestyle changes. At both the epidemiological and molecular levels, breast cancer is diverse. Many significant breast cancer risk factors have been discovered by clinical and epidemiological data, including age, family history, early menarche, and medical history; variables that are intangible or beyond our control. However, about 70% of breast cancers nowadays are caused by risk factors that may be altered or avoided. Obesity, lack of exercise, smoking, drinking, and nutrition, as well as other variables that may have a detrimental impact on a woman's hormonal environment, are among them. These important rate-limiting measures in the battle against breast cancer should not be ignored. As discussed in this review, significant advances in cancer biology have led to significant advancements in cancer early detection, therapy, and prevention in recent years. The growing emphasis on personalised treatment, as well as the combination of targeted and immunological therapies with current therapeutic techniques, holds potential for the cure of breast cancer. Drug resistance in breast cancer is a complicated clinical condition caused by a variety of molecular changes. Because chemotherapy is often used in conjunction with targeted treatments for the ER+ or HER2+ subtypes in clinical practice, targeted therapy-induced resistance may lead to chemo-resistance and vice versa. Treatment methods and therapeutics must be specially developed to address each distinct resistance mechanism in various clinical circumstances in response to every particular resistance mechanism. Early clinical trials are looking for drugs that target each route individually. Clinical studies

investigating tailored medication delivery methods are also underway in the meanwhile. These therapeutic agents may enter cells through receptor-mediated endocytosis, thereby bypassing typical drug resistance mechanisms such as drug efflux pumps, cell surface docking site mutations, and so on, allowing them to overcome drug resistance. The heterogeneity of breast cancer cells, on the other hand, poses major difficulties in terms of treatment response and may be a contributing factor in drug resistance. Tamoxifen, a selective oestrogen receptor modulator, is claimed to be used as a therapy for all stages of oestrogen receptor (ER)-positive breast cancer in men and women, thanks to its low risk of adverse effects. Notwithstanding major investments in prevention and treatment, breast cancer remains the primary cause of cancer mortality in women throughout the world. The existing therapeutic options are both expensive and have serious negative effects.

Drug repurposing, or finding new applications for existing therapies, has arisen as an unique drug development strategy. Repositioning existing, off-patent non-cancer medicines with established targets into newer indications is like repurposing outdated weaponry for a new war. The process of medication repurposing has been made easier thanks to developments in genomics, proteomics, and information computational biology. The repositioning method not only speeds up the medication development process, but it also results in more effective, less expensive, and safer medicines with fewer/known adverse effects. Alkylating compounds, anthracyclins, antimetabolites, CDK4/6 inhibitors, aromatase inhibitors, mTOR inhibitors, and mitotic inhibitors have all been repurposed for breast cancer therapy in the recent decade.

14. Conclusion and future perspectives

Medical experts are enthusiastic about the increasing management methods, but they are concerned that resources will be inadequate to get these therapeutics to advanced clinical trials. The difficulties are therefore to choose the most competent drugs to be examined, as well as the appropriate clinical trials to conduct such assessments. Over the last several years, new drugs targeting particular therapeutic targets have resulted in significant advances in the treatment of breast cancer. Resistance to systemic therapy (endocrine and others), expensive treatment, and limited availability of adequate cancer care in many countries remain challenges. We must continue to improve our available technology in order to provide proper guidance for those living with the disease, as well as those at high risk of developing it, and to develop new, more effective therapies in order to significantly improve the outcomes of breast cancer patients around the world. Individualising therapies offers the potential of helping patients through challenging treatment choices in order to enhance their long-term results. In this review, we have uncovered the most well-documented therapy options and potential technologies in the fight against breast cancer. We go through the benefits of medication repurposing for breast cancer treatment in depth in this article. We offered a number of medicines that were effectively repurposed for the treatment of breast cancer. Preclinical investigations have shown that a combination of chemotherapies and a medication repurposing strategy might produce promising results. The possibility of non-cancer drugs being studied for breast cancer in the future, as well as the obstacles and bottlenecks of drug repurposing, were also highlighted. As a result, we draw the conclusion that combining system biology and bioinformatics to select the most appropriate gene-protein-pathway-target-drug modelling has a high potential for providing more efficient, safer, and cost-effective chemotherapeutics for the treatment of even the most severe forms of breast cancer.

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Repurposing of Metformin as a Multifaceted and Multitasking Preventative and Treatment for Cancer

Raymond Chang

Abstract

Metformin is a cornerstone treatment of diabetes mellitus. Since 2005 when it has been first reported to reduce the risk of cancer in diabetics, a large number of preclinical and clinical studies have implicated its potential role as a preventative and adjunct therapy for a broad range of cancers. Whereas preclinical studies demonstrate its actions on a multitude of molecular pathways involving nearly all aspects of cancer development including metabolism, angiogenesis, apoptosis, autophagy, immunity, epigenetics, inflammation and crosstalk with the microbiome, other studies demonstrate its synergism with a range of anticancer modalities including chemotherapy, radiotherapy, immunotherapy, and targeted therapies. Furthermore, an increasing number of clinical studies not only confirm its preventative properties against cancers but have extended its potential for a possible adjunctive role in the neoadjuvant, adjuvant, maintenance and salvage therapies of cancer. This article intends to summarize the basic science that allows us to understand the complex multiple mechanisms of action of this remarkable multitasking molecule as well as review the recent meta-analyses that have summarized the clinical studies assessing the therapeutic efficacy of metformin for various cancers.

Keywords: metformin, diabetes, repurposing, cancer therapy

1. Introduction

Metformin is derived from the French lilac (also known as goat's rue or *Gallega Officinalis*), a medieval European medicinal herb that was first described as a diabetes treatment in a mid-17th century English treatise called *Culperper's Complete Herbal*, but it was not until 1957 that the French physician Jean Sterne formally patented metformin as a drug treatment for diabetes. The efficacy of metformin for type 2 diabetes mellitus (T2DM) has since been established and it was approved by the US FDA in 1995 as a treatment for T2DM. Meanwhile by the late 1980's, studies on the effects of metformin on insulin receptor binding on tumor cells led researchers to conceive that metformin's effect may potentially be applied for cancer management [1]. Separately, it has long been suspected that T2DM may be a risk for cancer with its cancer promoting effects believed due to hyperinsulinemia in T2DM, since insulin was believed to exert a mitogenic effect [2], thus it was simply logical to investigate the potential

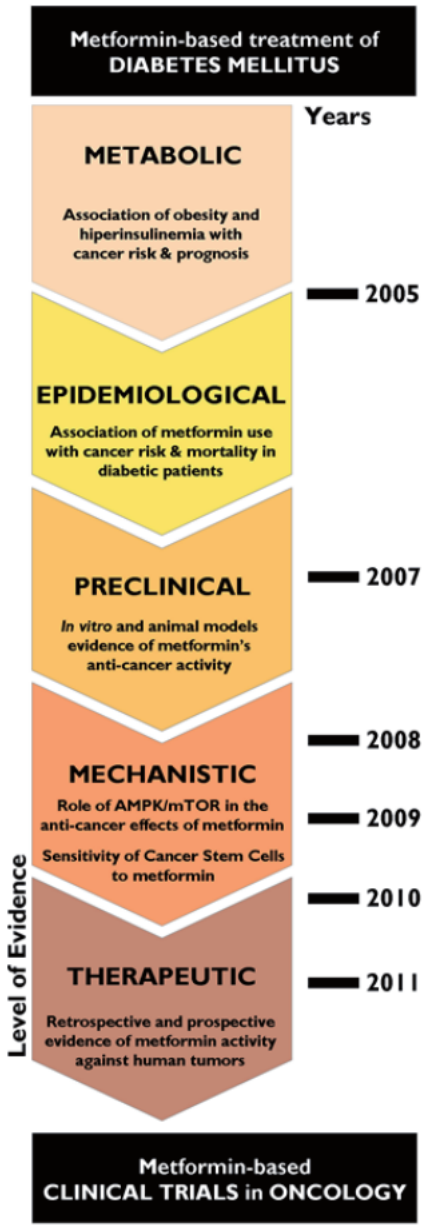

Figure 1.
Research development of metformin as anticancer agent: Since early epidemiologic reports suggesting metformin use in type 2 diabetes was associated with reduced cancer incidence, research evidence that metformin may be preventive and/or therapeutic for human cancers has expanded, with most of the molecular and clinical breakthroughs in metformin and cancer have taken place during the past decades, and hundreds of clinical trials are currently exploring metformin's potential in cancer. Source: [5], Licensed under CC BY 3.0.

benefits of an insulin lowering and hence counter-mitogenic anti-diabetic agent for its possible anti-cancer effects. By the early 2000's, studies have already established the potential benefits of metformin on hyperinsulinemia, obesity, hyperlipemia, hypertension, fibrinolysis, and endothelial dysfunction, with the expansion of the drug's potential applicability beyond T2DM to address weight gain, acanthosis nigricans, infertility and polycystic ovary syndrome [3]. In 2005, a landmark retrospective case control study by Evans et al. demonstrated that metformin exposure in T2DM was associated with the reduced risk of cancers [4] and further epidemiological studies

also corroborated that diabetics treated with metformin have a lowered incidence of cancer than those treated with other agents, leading to increasing calls for the use of metformin to reduce the risk of cancer. In the past decade, metformin has seen over 50 million prescriptions per year in the US alone and there has been a concurrent explosion of interest in metformin's anticancer effects with dozens of systematic reviews and meta-analyses performed and published on hundreds of cancer studies involving hundreds of thousands of patients and with hundreds of clinical trials on metformin and cancer currently actively recruiting. The development and expansion of research into metformin's anticancer activities in the past two decades from the bench to the clinic is illustrated below in **Figure 1**.

Given that this review is intended as a summary of current clinical evidence for the potential uses of metformin in the prevention and treatment of cancer, we will provide only a succinct synthesis of the thousands of preclinical studies on the biological mechanisms and molecular pathways that has been performed in the past two decades and focus our attention mostly on recent clinical evidence of metformin's efficacy as demonstrated by clinical studies.

2. Pleiotropic effects of metformin against cancer

The early days of laboratory research on metformin's anti-cancer mechanisms focused mainly on its metabolic effects on cell proliferation, which naturally follows from the initial use of metformin as a treatment for T2DM as a metabolic disorder. Eventually, it became gradually apparent that unlike modern day targeted therapies, metformin's anti-neoplastic bioactivity is broad ranged and pleiotropic, encompassing not only its established metabolic effects, but also involving antiangiogenic, anti-inflammatory, epigenetic, apoptotic and autophagic, and immunologic actions as well as effects on the microbiome and on cancer stem cells (CSCs) that all synergistically contribute to overall cancer prevention and control. Furthermore, within each category of its bioactivity, it further exerts multiple molecular actions, and it has thus become increasingly apparent that metformin could be properly conceived of as a multi-faceted multi-tasking molecule with direct and indirect actions against cancer. In summary, the anticancer effects of metformin is based on 1) its main action on cellular metabolism via the maintenance of plasma glucose and insulin levels, 2) targeted action against cancer cells with pleiotropic inhibitory effects on multiple pathways involved in cancer cell survival and metastasis, and 3) indirect anti-angiogenic anti-inflammatory as well as immunomodulatory effects and also its actions on the microbiome and CSCs. The complex pleiotropic nature of metformin effects on cancer is illustrated in **Figure 2**.

2.1 Metformin metabolic effects

To understand the metabolic impact of metformin on cancer, we must first recognize the intimate relationship between glucose energy metabolism and cellular proliferation as well as a unique propensity of cancer cells to utilize glucose anaerobically even in the presence of oxygen in contrast to non-cancer cells which utilize oxidative phosphorylation to generate energy. This phenomenon was first noted by Otto Warburg almost a hundred years ago, and subsequently termed the "Warburg effect" [7]. This altered energy metabolism of cancer cells may underline their proliferation, invasiveness, and chemoresistance and this altered metabolic pattern in cancer is regulated by oncogenic and tumor suppressor signals such as hypoxia inducible factor 1 (HIF-1), myelocytomatosis oncogene cellular homolog

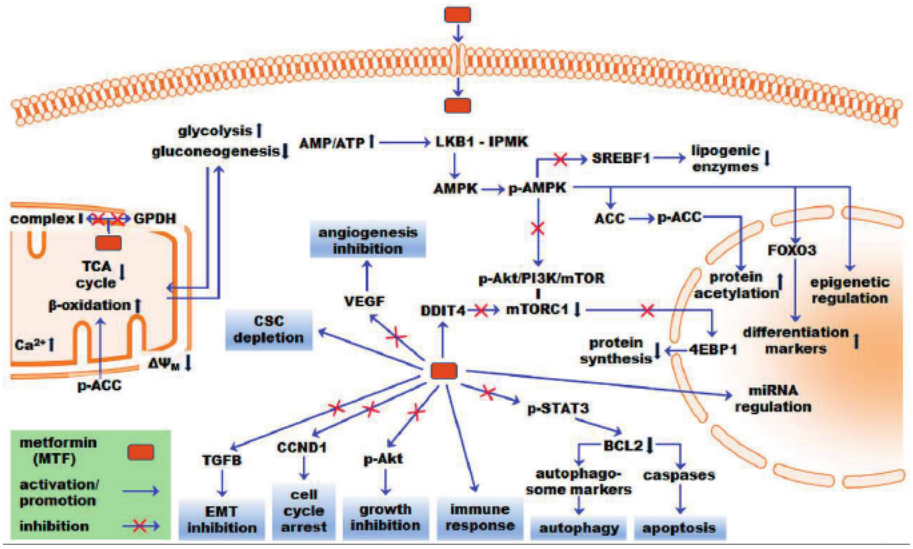

Figure 2.
Representation of some of the pleiotropic direct and indirect anticancer effects of metformin as illustrated by molecular and cellular pathways. Metformin effects key energy and metabolic processes such as the mitochondrial respiration (complex I), TCA cycle, fatty acid β-oxidation, gluconeogenesis, and glycolysis. Metformin affects the cell cycle, cell growth, immune response, autophagy, and apoptosis, angiogenesis and cancer stem cells. Abbreviations: 4EBP1, 4E-binding protein 1; ACC, acetyl-CoA carboxylase; AKT, AKT serine/threonine kinase 1; AMPK, AMP-activated protein kinase; BCL2, apoptosis regulator, BCL2; CCND1, cyclin D1; CSC, cancer stem cell; DDIT4, DNA damage inducible transcript 4; EMT, epithelial-to-mesenchymal transition; FOXO3, forkhead box O3; GPDH, glycerol-3-phosphate dehydrogenase; IPMK, inositol polyphosphate multikinase; LKB1, liver kinase B1; miRNA, micro RNA; mTORC1, target of rapamycin complex 1; SREBF1, sterol regulatory element binding transcription factor 1; STAT3, signal transducer and activator of transcription 3; TCA, tricarboxylic acid; TGFB1, transforming growth factor beta 1; VEGF, vascular endothelial growth factor. Phosphorylated molecules are indicated by a prefix p. source: [6], Licensed under CC BY 4.0.

(Myc), p53, and the phosphoinositide 3 kinase (PI3K)/AKT8 virus oncogene cellular homolog (Akt)/mammalian target of rapamycin (mTOR) pathways.

Metformin's main pharmacologic action is the reducing elevated plasma glucose is largely due to the improvement in hepatic insulin resistance leading to a reduction in hepatic glucose output from gluconeogenesis, increases glucose uptake in muscle, decreased absorption of sugar from the intestines, and improved insulin sensitivity, mainly via activation of a cellular energy sensor known as AMP-activated protein kinase (AMPK). The major downstream target of AMPK is mTOR, which is very important in cellular growth processes and cancer dynamics, and mTOR is inhibited by AMPK [8]. Since glucose metabolism is at the center of the metabolic derangement that is a hallmark of cancer cells, and metformin chiefly targets glucose metabolism, it follows that the altered metabolic pathway may be a target by metformin for cancer prevention or therapy.

It is through its main effects above on metabolism and cellular energetics that metformin can attenuate cancer cell proliferation (See **Figure 3**). Furthermore, these metabolic effects in turn impact the immune system, epigenetics, inflammation, cellular apoptotic and autophagic pathways as well as the microbiome and CSCs which all play a role in cancer development.

2.2 Metformin immuno-modulatory effects

The immune system participates broadly in the prevention and control of cancer and interacts with biological pathways of metabolism and inflammation,

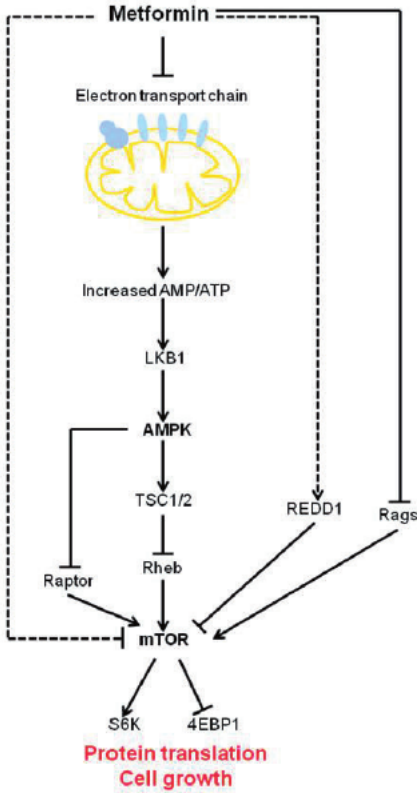

Figure 3.
The effect of metformin in suppressing cancer cell growth via metabolic pathways. Metformin inhibits complex I of the electron transport chain, which leads to increased AMP/ATP ratio and activation of AMPK by LKB1. Activated AMPK subsequently inhibits mTOR and its downstream targets by the following two pathways: 1. AMPK stabilizes TSC1/2, which inhibits Rheb, an activator of mTOR; 2. AMPK inhibits mTOR binding protein raptor. Metformin directly inhibits mTOR by up-regulating REDD1 and suppressing rags. AMPK, AMP-activated protein kinase; Rheb, Ras homolog enriched in brain; LKB1, liver kinase B1; REDD1, regulated in development and DNA damage response 1; TSC, tuberous sclerosis complex; rags, rag GTPases; mTOR, mammalian target of rapamycin; 4EBP1, eukaryotic initiation factor 4E binding protein 1; S6K, S6 kinase. Source: [9], Licensed under CC BY 3.0.

and metformin again acts in a multifaceted fashion to bolster immunity against cancer with effects on almost every aspect of the immune system, especially with reference to cancer immunty (**Figure 4**). One of metformin's actions is the enhancement of CD8+ T lymphocytes and rescues them from exhaustion. CD8+ T cells which is one of the key components in cellular immunity against tumors, as these cells can expand and transform into effector cytotoxic T lymphocytes (CTL) which targets cancer. This phenomenon of the rescue of exhausted CD8+ T lymphocytes has been confirmed *in vitro* in leukemia, melanoma, renal cell carcinoma, non–small-cell lung carcinoma (NSCLC), gastrointestinal carcinoma, and breast cancer. Also, metformin-induced activation of AMPK as one of its main metabolic actions mentioned above promotes immune check-point programmed death ligand 1 (PD-L1) degradation, which allows CTL-mediated tumor cell death [11]. Additionally, metformin can also enhance local as well as systemic cytokine responses to tumors [12]. Furthermore, metformin also has indirect effects on the immune system via its influence on the microbiome and its anti-inflammatory effects, which has been reviewed exhaustively and is briefly summarized below.

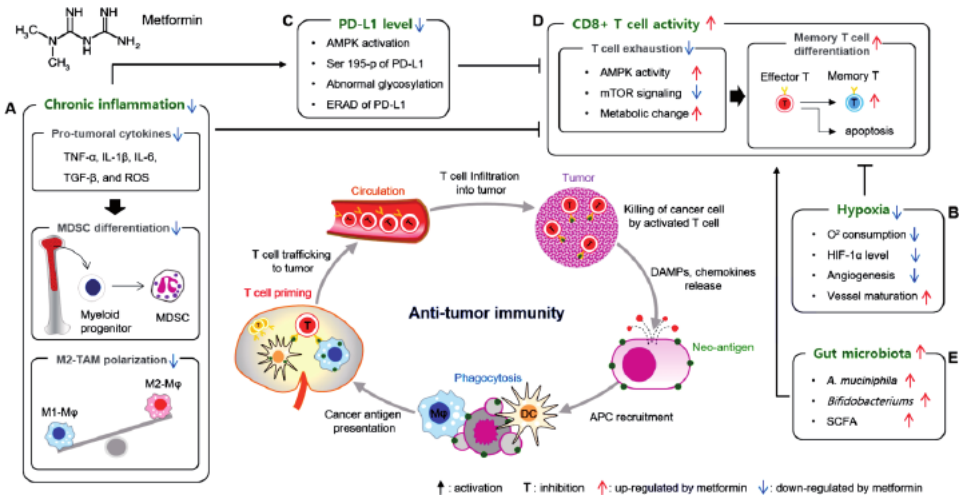

Figure 4.
Metformin effects related to anticancer immunity. Metformin indirectly increases T-cell activity by negatively regulating (a) chronic inflammation, (B) hypoxia, and (C) PD-L1 levels that inhibit T-cell activity. Metformin directly relieves T-cell exhaustion by means of metabolic reprogramming of TIL and promotes memory T-cell differentiation (D). Metformin shifts the profile of gut microbiota more favorably to T-cell immunity (TAM) tumor-associated macrophages (E); (Mφ) macrophages; (MDSC) myeloid-derived suppressor cells; (T) T-cell; (DAMPs) damage-associated molecular patterns; (APC) antigen presenting; (SCFA) short-chain fatty acid. Source: [10], CC BY-NC 3.0.

2.3 Metformin effects on the microbiome

Whereas science has become increasingly aware of the central role the gut microbiome plays in health and diseases including cancer, particularly via its effects on the immune system [13], metformin's beneficial role on host metabolism has also been found to be in part related to the microflora in the gut. The microbiome modulates our immune system and inflammatory response and both of these are key factors in determining cancer development and are associated with inflammatory immune response [14] highlights the crosstalk between metformin effects on metabolism, immunity, inflammation and the microbiome, which in turn can modulate cancer biodyamanics, and part of the mechanisms involved in this complex interplay is illustrated in **Figure 5** below.

2.4 Metformin anti-inflammatory effects

Inflammation effects on cancer promotion is well known. In 1863, Rudolf Virchow first proposed the role of inflammation in cancer based on the observation of leukocytes in cancerous tissue. Subsequently, accumulated evidence has identified inflammation both as a cause and result of malignancy [16], with numerous studies in past decades implicating chronic inflammation in the promotion of malignancy [17] (**Figure 6**). Not surprisingly then, given the T2DM's known association with chronic low-grade subclinical inflammation which is part and parcel of its the insulin resistance that is its hallmark [19], and metformin's effects on the immune and metabolic systems, that metformin must also modulate the inflammatory response. This connection has been well demonstrated by animal experiments where metformin treated rodents reveal dampened pro-inflammatory pathways nuclear factor k B (NF-k) and Jun N-terminal kinase (JNK) and increased anti-inflammatory cytokine IL-10 [20].

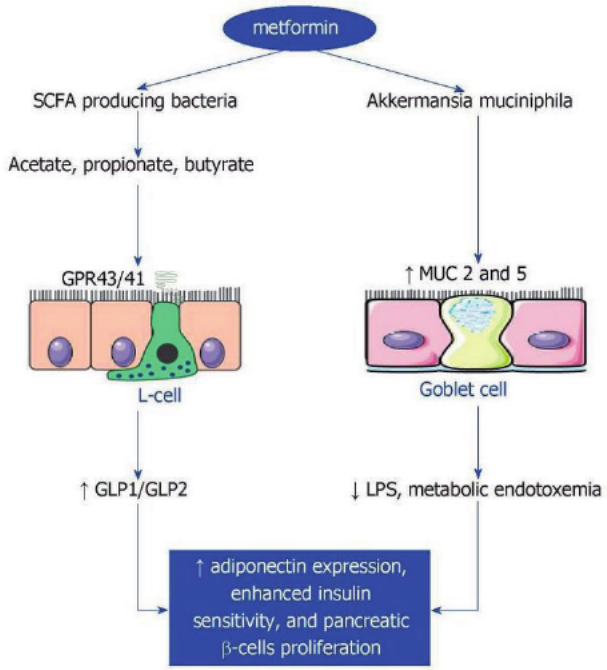

Figure 5.
Crosstalk between metformin action and gut microbiota. GLP1: Glucagon-like peptide-1; GLP2: Glucagon-like peptide-2; LPS: Lipopolysaccharide; SCFA: Short-chain fatty acid. Source: [15], Licensed under CC BY-NC 4.0.

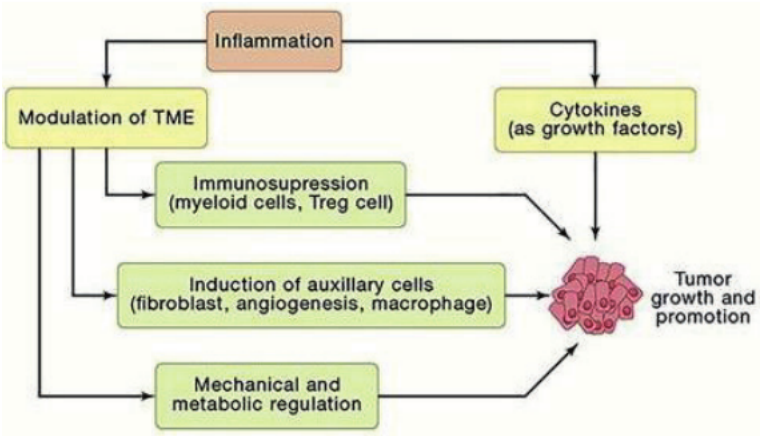

Figure 6.
Inflammatory cytokines released by immune cells within the tumor microenvironment has a direct effect on pre-malignant and cancer cells by increasing their proliferation and resistance to cell death and stresses thus directly promoting tumor growth and progression. Additionally, inflammatory signals can suppress anti-tumor immunity via action of regulatory T-cells, myeloid cells and enhance other cancer promoting cells (such as fibroblasts, myeloid cells and endothelium of new blood vessels); altogether, these inflammation driven changes also significantly contribute to tumor growths and progression. TME: Tumor microenvironment, Treg: Regulatory T cells. Source: [18], Licensed under CC BY 3.0.

2.5 Metformin epigenetic effects

Epigenetics is the genomic mechanism that reversibly modulates gene expression independent of DNA sequences. Epigenetic processes which allow for the gene modulatory effect involve DNA methylation, histone modification,

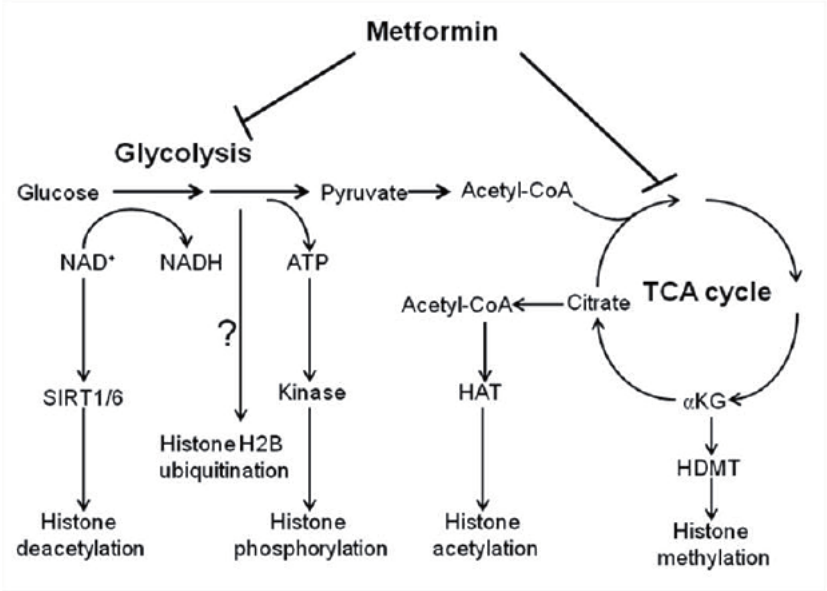

Figure 7.
Schematic of histone modifications via metabolic effects of metformin. Glycolysis determines the NAD⁺/NADH ratio, which affects the activity of histone deacetylases to reduce histone acetylation. Source: [9], Licensed under CC BY 3.0.

the readout of these modifications, chromatin remodeling and the effects of noncoding RNA all of which affects cellular activities such as growth and differentiation. Thus, epigenetics can in one sense be conceived of as a master switch of cancer biological processes. Recently, there has been growing interest in epigenetic targeting as a promising therapeutic option for cancer [21]. And since cellular metabolism is tightly linked to epigenetic modifications, it is again not surprising that metformin as a modulator of cellular metabolism may also possess significant epigenetic effects mainly via histone modification (**Figure 7**), which in turn is another avenue whereby metformin may exert its anti-cancer effects [9].

2.6 Metformin apoptotic and autophagic effects

Both apoptosis or programmed cell death and autophagy are important catabolic and tumor-suppressive pathways that control cell survival and cell death and are thus increasingly important therapeutic targets in cancer [22]. While apoptosis involves cellular suicide and cell death pathways, autophagy involves recycling and degradation of cellular waste which if maladapted and excessive can also lead to cell death and there is significant cross-talk between these two pathways [23]. In cancer biology, autophagy is cancer suppressive as it facilitates the degradation of oncogenic molecules thus pre-empting the development of cancers, while apoptosis leads to cellular suicide and limits the survival of cancer cells. As a result, defective or inadequate autophagy or apoptosis can both lead to cancer. The complexity of the crosstalk between the apoptosis and autophagy is illustrated in **Figure 8**.

In the case of these pathways, metformin has been shown to promote apoptosis in a variety of cancers via various biological pathways [24] while also promoting autophagy [25] as two other dimensions of its anti-cancer bioactivity.

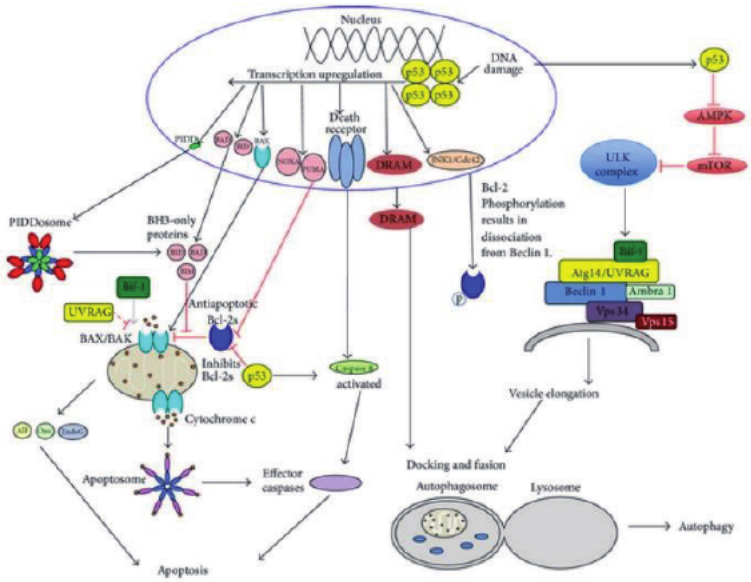

Figure 8.
Complex crosstalk between autophagy and apoptosis pathways. Various proteins involved at the different points of crosstalk are shown and labeled. Lines denote interactions or processes, with solid lines corresponding to intrapathway processes and dashed lines corresponding to inter-pathway connections. Red lines denote inhibitory interactions, while lines with arrows indicate facilitating interactions. Source: [24], Licensed under CC BY 3.0.

2.7 Metformin effects on cancer stem cells

CSCs were only identified in the 1990s, and they have been hypothesized to persist in tumors as a distinct cell population capable of self-renewal and maybe responsible for cancer relapse and metastasis by giving rise to new tumors. These CSCs are also believed to be resistant to traditional chemotherapy and radiation. A complex regulatory network consisting of microRNAs and Wnt/β-catenin, Notch, and Hedgehog signaling pathways control the properties of CSCs. Therefore, the development of specific therapies targeting and its regulatory pathways is another avenue for improved cancer treatments to prevent relapse and metastases, and improve survival [26]. In this regard, metformin has been reported to target CSCs perhaps via blunting of the Warburg effect and consequently down-regulates their growth. In animal studies, it has been found that metformin exposure was associated with a ~ 2-fold reduction in ovarian CSCs and increased in chemotherapy response and translational studies completed as part of a multi-center phase 2 clinical trial was able to demonstrate a 2.4-fold CSC reduction as well as improved survival in ovarian cancer patients [27].

2.8 Metformin's antiangiogenic effects

Angiogenesis is the process where a tumor can induce its own blood supply via neovascularization to enhance its own nutrient source as well as increase its propensity to metastasize. It follows that antiangiogenesis which involves the suppression of vascular supply to tumors may be an effective method of cancer control as initially proposed by Folkman [28]. In this regard, preclinical studies with metformin have reported that it indirectly modulates tumor angiogenesis most likely via metabolic pathways affecting proangiogenic signals. As an example, metformin is known to decrease HIF-1α stability in cancer cells, reducing the expression of HIF-1

targeted genes and thus resulting in smaller tumor vessel size, reduced microvessel density and slower tumor growth [29]. Another murine experiment analyzing angiogenesis in a matrigel plug model found that metformin treatment lead to a decrease in angiogenesis [30].

3. Deployment strategies for metformin in cancer

Metformin can be used tactically under various scenarios against cancer. It can be used as standalone or in combination with other agents for the primary or secondary prevention of cancer [31], as neoadjuvant or adjuvant cancer therapy [32], as maintenance therapy or salvage therapy, or to reduce chemoresistance or enhance radiosensitivity [33] as well as for the reduction of side-effects or complications [34]. Notably, metformin is usually deployed as an adjunct but not as a sole agent except in the case of primary prevention. Since it has such low toxicity and multifaceted mechanisms of actions, it is usually integrated with other treatment agents and modalities under other scenarios besides primary prevention. The key feature of metformin that allows this combinatorial deployment is its low toxicity and its synergism with various other agents and modalities, as it has been demonstrated both *in vitro* and *in vivo*.

3.1 Metformin synergies with other anticancer agents and modalities

Notably, synergisms with metformin has been reported with numerous anticancer agents and modalities including chemotherapy [35], targeted drugs [36], and radiotherapy [37]. In the past ten years alone, metformin synergism with chemotherapies pemetrexed [38], temozolomide [39], cisplatin [40], gemcitabine [41], paclitaxel [42], 5FU [43], vincristine [44] with targeted agents erlotinib against non-small cell lung cancer [45], imatinib against colon cancer [46], gefitinib against bladder cancer [47], trastuzumab against human epidermal growth factor receptor 2 (HER2) positive breast cancer [48], celecoxib against NSCLC [49], regorafenib against liver cancer [50], with everolimus as neuroendocrine cancers [51]; and other anticancer agents such as with nelfinavir against cervical cancer [52], propranolol against breast cancer [53], 2-deoxyglucose against ovarian cancer [54], arsenic trioxide against cholangiocarcinoma [55], and with natural compounds epigallocatechin-3-gallate [56], curcumin [57], berberine [58], resveratrol [59].

What is interesting is that different biological mechanisms may be responsible for the efficacy of metformin's combinatorial effects depending on the specific combination. For example, regulation of lipid synthesis may underlie metformin enhancement of taxanes, pro-apoptotic mechanisms could account for its synergy with cisplatin, AMPK/mTOR signaling maybe significant when combined with hormonal drugs, and suppression of HIF-1, P glycoprotein (p-gp) and multidrug resistance-associated protein 1 (MRP1) expression is thought to be responsible for metformin's synergy with anti-metabolites [60]. In the case of targeted agents such as the epidermal growth factor receptor (EGFR) inhibitor gefitinib against NSCLC where a Chinese study on diabetic NSCLC patients on gefitinib demonstrated significantly improved response rate, disease control rate, median progression free survival (PFS) and median overall survival (OS) compared with patients controls (70.5% vs. 45.7%, $P = 0.017$; 97.7% vs. 80.4%, $P = 0.009$; 19 months vs. 8 months, $P = 0.005$; 32 months vs. 23 months, $P = 0.002$, respectively) [61]. Separately, metformin combination with m-TOR inhibitor everolimus in patients with advanced pancreatic neuroendocrine tumors showed improved median PFS of patients treated with the combination vs. control (median PFS, 20.8 months;

hazard ratio, 0.49; 95% confidence interval (CI), 0.34–0.69; $P < .0001$), suggesting that metformin may sensitize everolimus in these patients [62]. As far as combination with antibody treatments go, a randomized phase II study of metformin plus bevacizumab-based chemotherapy in advanced or metastatic NSCLC patients resulted in a 47% (95% CI, 25%–88%) one-year PFS in patients on metformin, which is much improved over a historical control of 15%. Median overall survival of 15.9 months of metformin treated patients was also improved over control arm of 13.9 months [63]. Furthermore, metformin in combination with immune checkpoint inhibitors (ICI) has received much recent attention as ICI is increasingly being deployed in cancer treatments. A retrospective review of 50 NSCLC patients receiving ICIs as second or third line therapy with or without metformin showed higher overall response rate, disease control, median OS and PFS in the metformin group (41.1 vs. 30.7%, $P = 0.4$; 70.5 vs. 61.6%, $P = 0.5$; 11.5 vs. 7.6 months, $P = 0.5$ and 4.0 vs. 3.0 months, $P = 0.6$, respectively) [64]. Very recently, several significant trials have been launched to further investigate the role metformin may have in combination with ICI's, including a metformin-nivolumab combination in patients with NSCLC (NCT03048500), a phase I trial investigating the combined effect of metformin and another anti-PD-L1 antibody durvalamab in head and neck squamous cell carcinoma (NCT03618654), a phase I trial of metformin in combination of the anti-PD-1 antibody pembrolizumab in advanced melanoma (NCT03311308), and a phase II trial combining metformin with nivolumab in stage IV colorectal cancer that has not responded to previous treatment (NCT03800602).

The use of metformin under various scenarios against cancer has been best studied clinically for primary prevention and in the neoadjuvant setting and some of the relevant data is summarized below.

3.2 Metformin for primary prevention of cancer

Cancer prevention is the earliest role that metformin was hypothesized to play in the disease as it was Evans' original 2005 retrospective case–control study demonstrating metformin's involvement in reducing cancer risk in T2DM that highlighted its potential for cancer [4]. Subsequently, a confirmative cohort study of T2DM with metformin followed in which the frequency of cancer was significantly lower in patients receiving metformin versus controls who had never received metformin, after adjusting for body mass index, hemoglobin A1C, smoking and the use of other drugs [65], a finding that was subsequently repeatedly confirmed. Indeed, meta-analyses have demonstrated that metformin is associated with a decreased risk of breast, colon, liver, pancreas, prostate, endometrium and lung cancer across meta-analyses [31] suggesting that people with T2DM receiving metformin demonstrate a lower risk and improved outcomes with most common cancers; more specifically one meta-analysis found that metformin-treated T2DM patients had a 31% reduction in the incidence of cancer and a 34% reduction in cancer mortality after adjusting for body mass index [66].

3.3 Metformin in neoadjuvant treatment

Neoadjuvant effects of metformin in combination or alone has been clinically explored in several cancers types. In one study of two hundred eighty-five patients with esophageal adenocarcinoma treated with concurrent chemoradiation followed by esophagectomy, complete remission (CR) was higher in T2DM patients taking metformin (34.5%) compared to those who are not (4.8%, $P = 0.01$) as well as non-diabetic patients who are not on the drug (19.6%, $P = 0.05$) and furthermore the CR rate was found to be related to metformin dose, with ≥1500 mg per day associated

with a higher CR rate [67]. In a separate study of diabetic rectal cancer patients undergoing neoadjuvant chemoradiotherapy, those on metformin experienced better tumor responses ($P = 0.002$), pathologic complete remission ($p = 0.037$), and N downstaging ($P < 0.001$) as well as experienced improved cancer specific survival and lower risk of recurrence [68]. Separately, women with endometrial cancer on neoadjuvant metformin 850 mg twice daily for an average of 20 days between diagnosis and surgery had reduced cell proliferation per Ki-67 expression, compared to the untreated [69]. A similar biomarker based on a "window of opportunity" assessment of metformin 500 mg three times daily for a median duration of 18 days in non-diabetic breast cancer also demonstrated that short-term preoperative metformin resulted in both clinical and cellular changes including a significant decrease in the Ki-67 proliferation index from 36.5 to 33.5% ($P = 0.016$) [70]. Separately and perhaps more significantly, a study involving early-stage breast cancer assessing remission rates after neoadjuvant therapy among metformin vs. non-metformin users found a significant difference in CR of 24% in the metformin group, 8.0% in the non-metformin group, and 16% in the non-diabetic group, with metformin use independently predictive of response (OR 2.95; $P = 0.04$) after adjustment for diabetes, body mass index, age, stage, grade, receptor status, and neoadjuvant chemotherapy use by multivariate logistic regression [71].

4. Systematic reviews and meta-analyses on metformin clinical outcomes in various cancers

Since metformin is so versatile and has been studied in a wide variety of settings from the laboratory to bedside, and since this review is intended to focus on the clinical deployment of metformin, it is thus useful to have a summary perspective of its potential usefulness in cancer by reviewing clinical results as recently meta-analyzed for various cancers.

4.1 Bladder cancer

A review of 9 retrospective cohort studies with 1,270,179 patients did not reveal a benefit from metformin in preventing bladder cancer (Hazard ratio (HR) = 0.82, 95% CI = 0.61–1.09; $P = .17$). However, metformin intake was associated with an improved recurrence-free survival (HR = 0.55, 95% CI = 0.35–0.88; $P = .01$), progression-free survival (HR = 0.70, 95% CI = 0.51–0.96; $P = .03$), as well as cancer-specific survival (HR = 0.57, 95% CI = 0.40–0.81; $P = .002$) [72].

4.2 Breast cancer

There have been a number of studies relating to metformin's effect on biomarkers in breast cancer patients and it has been shown that metformin therapy reduced the levels of insulin, sex hormones and sex hormone-binding globulin, Ki67, caspase-3, p-Akt, obesity, CRP, blood glucose and lipid profile overall [73]. More, in a clinical trial to examine the clinical and biological effects of neoadjuvant metformin on patients with breast cancer, non-diabetic women with untreated breast cancer given 500 mg of metformin three times daily for ≥2 weeks exhibited decreased insulin receptor expression ($P = 0.04$), phosphorylation status of protein kinase B /Akt, extracellular signal-regulated kinase 1/2, AMPK and acetyl coenzyme A carboxylase ($P = 0.0001$, $P < 0.0001$, $P < 0.005$ and $P = 0.02$, respectively) in tumors correlating with decreases in tumor cell proliferation and increases in apoptosis [74]. In T2DM patients with breast cancer, a 2018 meta-analysis of eleven

studies of all-cause mortality found a 45% risk reduction was observed for all-cause mortality (HR = 0.55; 95% CI 0.44–0.70) and concluded that metformin may improve overall survival in this patient subset [75]. Separately in another review, 7 observational studies showed significantly reduced breast cancer risk among T2DM patients on metformin OR = 0.83 (CI 0.71–0.97) [76]. Separately, in a sub-study involving over four hundred diabetic patients in the large phase 3 ALTTL trial of Her2+ breast cancer patients, Her2+ and estrogen receptor positive breast cancer cases on metformin experienced had improved disease free survival, metastasis free disease survival and overall survival over those patients not on metformin over a median of four and a half years [77]. However despite the vast amount of preclinical and epidemiologic data on its benefits in breast cancer, there are no trials in non-diabetic breast cancer patients to date which have unequivocally demonstrated a clinical benefit of metformin.

4.3 Colon cancer

Ng et al. from Singapore found 58 studies that provided incidences of colorectal adenoma and cancer and cancer survival outcomes and found that metformin significantly lowered the risk of colorectal adenoma (RR 0.77, CI 0.67–0.88, $P < 0.001$), advanced adenoma (0.61, CI 0.42–0.88, $P = 0.008$) and colorectal cancer (RR 0.76, CI 0.69–0.84, $P < 0.001$) respectively. Overall cancer survival (HR 0.6, CI 0.53–0.67, $P < 0.001$), even among metastatic cases was also higher among metformin users (HR 0.77, CI 0.68–0.87, $P < 0.001$), and it was concluded that metformin significantly reduces colorectal adenoma and cancer incidence as well as enhanced colorectal cancer survival at all stages [78].

4.4 Endometrial cancer

In 19 studies reviewed in 2017, metformin used reversed atypical endometrial hyperplasia to normal, and decreased cell proliferation from 51.94% (CI = 36.23% to 67.46%) to 34.47% (CI = 18.55% to 52.43%) [79], while separately, a review of seven studies showed that metformin could significantly improve overall survival of in endometrial cancer (HR = 0.61, 95% CI 0.48–0.77, $P < 0.05$) and reduce their recurrence risk (OR = 0.50, 95% CI 0.28–0.92, $P < 0.05$) [80], whereas another review of six retrospective cohorts if 4723 endometrial cancer cases demonstrated that metformin use was associated with a significant reduction in overall mortality in comparison with not using metformin (adjusted HR 0.64, 95% CI 0.45–0.89, $P = 0.009$) irrespective of diabetic status [81], and these results corroborated the improved overall (HR, 0.58; 95% CI, 0.45–0.76; $P = 0.207$) as well as progression free survival (HR, 0.61; 95% CI, 0.49–0.76; $P = 0.768$) found in another review of 6242 patients from fourteen studies [82].

4.5 Lung cancer

An analysis of 13 observational studies found lung cancer incidence to be reduced in diabetic patients on metformin vs. no metformin (RR = 0.89; 95% CI, 0.83–0.96; $P = 0.002$) [83]. A separate meta-analysis found six studies comparing metformin usage and non-metformin usage significantly improved overall survival in diabetic patients with NSCLC [pooled HR =0.87 (0.77–0.99), $P = 0.04$] [84]. Especially noteworthy was an ambitious prospective clinical trial conducted by Marrone et al. which studied non-diabetics with advanced or metastatic NSCLC receiving platinum-based doublet chemotherapy and bevacizumab with or without metformin 1000 mg twice daily followed by maintenance

therapy with bevacizumab and metformin combined or bevacizumab alone and showed a significant clinical benefit in PFS (9.6 vs. 6.7 months) with the addition of metformin [63].

4.6 Pancreas cancer

A review of seventeen studies involving 36791 participants study has evidenced a significant association of metformin adjuvant treatment in pancreas cancer with overall survival benefit (HR = 0.88, 95% CI = 0.80–0.97) especially in Asians, those with early stage disease and those undertaking surgery [85]. In terms of overall survival with metformin use in pancreas cancer, a study of 8 retrospective cohort studies and 2 randomized clinical trials representing 3,042 patients revealed overall survival to be improved with metformin (meta-HR = 0.79; 95% CI: 0.70, 0.92, $P < 0.001$) [86].

4.7 Prostate cancer

In a systematic review involving eleven studies with 877,058 patients, the odds ratio of metformin use for reducing prostate cancer was estimated at 0.89 (95%CI: 0.67–1.17) and it was concluded that metformin consumption reduced the risk of prostate cancer, although the result was not statistically significant [87]. Separately, a review of eight studies on diabetic patients with prostate cancer found no metformin use was associated with an increased risk of cancer recurrence (RR, 1.20; 95% CI, 1.00–1.44) [88], which concurs with another review of eight retrospective cohort studies and one nested-case–control study, metformin was found to be associated with a reduced risk of biochemical recurrence (pHR: 0.82, 95% CI 0.67, 1.01, $P = 0.06$) [89]. Finally, a large review of 30 cohort studies, including 1,660,795 prostate cancer patients revealed that metformin treatment compared with no treatment improved overall, prostate cancer specific, and recurrence free survival (HR = 0.72, 95% CI: 0.59–0.88, $P = 0.001$; HR = 0.78, 95% CI: 0.64–0.94, $P = 0.009$; and HR = 0.60, 95% CI: 0.42–0.87 $P = 0.006$, respectively) [90].

4.8 Ovarian cancer

One review of 13 studies involving ovarian cancer incidence and prognosis revealed metformin use to be associated with a lower incidence (pooled OR 0.76, 95% CI 0.62 to 0.93, $P = 0.008$) as well as improved prognosis (pooled OR 0.55, 95% CI 0.36 to 0.84, $P = 0.006$) [91].

4.9 Other cancers

Metformin is also increasingly studied or planned in less common cancers, such as glioblastoma, thyroid cancer, and non-Hodgkin's lymphoma. The recent study on newly diagnosed glioblastoma showed that temozolomide plus memantine, mefloquine, and metformin are feasible as an adjuvant therapy [92]. One planned phase 1b/2 clinical trial of metformin and chloroquine was recruiting patients with IDH1-mutated or IDH2-mutated solid tumors, including glioma [93]. In another recent retrospective study from Korea, cancer preventative effects of metformin on thyroid cancer were observed in individuals with T2DM on long duration or higher doses of the drug [94]. Separately, a trial in head and neck squamous cell cancer patients revealed metformin to inhibit cancer by enhancing apoptosis, and increasing cellular immune infiltration of the cancer [95]. In non-Hodgkin's lymphoma,

a retrospective analysis of looking at T2DM patients treated with standard therapy found improved progression-free survival and overall survival compared to control not taking metformin [96].

5. Discussion

Any discussion of a therapeutic agent is incomplete without covering its toxicity, side-effects and drug interactions. In this regard, metformin is probably one of the safest drugs in use, especially when compared with standard anti-cancer agents in its context as a potential cancer preventative or therapeutic. With its long history of widespread use, its pharmacokinetics and toxicity profile are well established. The most common side-effect is mild to moderate gastrointestinal discomfort or diarrhea which is usually self-limited and can be minimized if metformin is taken with food, while its most serious side-effect of lactic acidosis usually due to overdose is relatively rare, occurring once per 100,000 years of use or 3 case per 1,000,000 after long term treatment [97]. As in the case of all medications, it should be dispensed carefully in elderly patients and in those with impaired renal, cardiac, and hepatic function. For practical purposes, it needs to be emphasized that metformin as an antidiabetic and as monotherapy does not cause hypoglycemia or weight gain, unlike insulin or sulfonylureas. For cancer, because of its very common use in diabetics, it has practically seen combined use with most oncologic agents in the diabetic cancer patient and remarkably no serious interactions with standard cancer anti-cancer agents have been reported. The minimum toxic dose of metformin is not well defined, but rare case reports of severe toxicity has only been reported after ingestion of 25 to 35 grams of metformin by adults.

A treatment for any condition is ideal if it relatively non-toxic and scientifically well evidenced, as well as low in cost and convenient to administer. Metformin fits all the above criteria. It is apparent from our review that metformin has ample scientific evidence from bench to the bedside as a repurposed drug for cancer. In fact, it is safe to say that it is currently the most well evidenced repurposed drug for cancer. Also, its wide-spread and decades of experience of clinical use and low observed toxicities alone or in combination with other agents, as well as very low cost also marks it as an optimal therapeutic agent. Finally, the versatility it possesses against various cancers and its applicability from prevention to treatment further distinguishes it as an ideal or model repurposed drug for cancer.

Of course, there remains limitations and challenges to metformin's use as an anticancer. The first obstacle we have in translating *in vitro* results of metformin to the clinical arena revolves around its dosage. The usual dosage of metformin in cancer trials is the same range as that prescribed normally for T2DM which is from 1000 mg – 2000 mg per day. Treatment is usually started at the lower dose with dose escalations weekly to the maximum dose which means starting at 500 mg of the immediate release version twice daily or 850 mg of the extended release versions once daily, with 500 mg increments weekly as tolerated, to a maximum of 2000–2550 mg per day for either immediate or extended release versions. It may not be apparent at first glance, but the concentration of metformin at 10–100 microM when used clinically at 1000-2000 mg per day is much less than the concentration of >2–5 mM demonstrated for its anti-cancer effects *in vitro* where metformin was usually experimented at concentrations between 5 to 20 mM, which is 2 000–10 000 times more concentrated than achieved with clinical dosing [98]. Fortunately, many clinical studies still yielded positive results at the much lower metformin in concentrations achieved with clinical dosing, but it may also explain why the

clinical results of metformin in cancer may not be as dramatic as demonstrated in preclinical studies, and why it is never intended to be used as monotherapy for cancer treatment. Related to dosing is a possible dose-dependent effect of metformin on cancer risk [99], which raises the question of attempting higher doses of metformin in future clinical trials of metformin in cancer, this while taking into account that there are no cases of acute metformin overdose leading to death found in which patients with a peak serum metformin concentration is under 50 microg/mL [100]. Beyond dosing, another issue with the literature to date on metformin and cancer is that most of the clinical studies so far are retrospective that mainly involve observations in the T2DM patient population and thus subject to selection bias. However, many cohort studies in the non-diabetic is planned, and despite methodological limitations, it is apparent that the overwhelming evidence so far is in favor of potential benefits and a high benefit to risk and benefit to cost ratios for metformin's application in cancer.

6. Conclusion

As an old repurposed drug, metformin is inexpensive and generic and its research is thus carried out usually without industry support. Despite such challenges, it is heartening that overall preclinical and clinical results is overwhelmingly suggestive of a protective effect from metformin against various stages of a wide spectrum of cancers. Moreover, there are over three hundred registered clinical trials on metformin and cancer internationally as of mid-2020, of which approximately one third are actively recruiting. The trials involve metformin for pre-cancers, early stage as well as metastatic solid tumors, alone or in combination with other interventions including chemotherapy, radiotherapy, hormone therapy, immunotherapy (ICIs), targeted agents, statins, aspirin, doxycycline, nelfinavir, melatonin, disulfiram, vitamin C, diet in diabetics and non-diabetics. We thus look forward for the further establishment of metformin as an ideal repurposed agent for cancer prevention and treatment.

Acronyms and abbreviations

Akt	AKT8 virus oncogene cellular homolog
AMPK	AMP-activated protein kinase
CI	confidence interval
CR	complete remission
CSC	cancer stem cell
CTL	cytotoxic T lymphocytes
EGFR	epidermal growth factor receptor
GPDH	glycerol-3-phosphate dehydrogenase
HER2	human epidermal growth factor receptor 2
HIF	hypoxia inducible factor; HR: hazard ratio
hs-CRP	C-reactive protein
ICI	immune checkpoint inhibitor
JNK	Jun N-terminal kinase
mTOR	mammalian target of rapamycin
MRP-1	multidrug resistance-associated protein 1
Myc	myelocytomatosis oncogene cellular homolog
NAD+/NADH	nicotinamide adenine dinucleotide (oxidized)/nicotinamide adenine dinucleotide + hydrogen

NF-kB	nuclear factor k B
NSCLC	non-small cell lung cancer
OS	overall survival
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
p-gp	P glycoprotein
PI3K	phosphoinositide 3 kinase
PFS	progression free survival
T2DM	type 2 diabetes mellitus
Treg	regulatory T cell

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Ivermectin: Potential Repurposing of a Versatile Antiparasitic as a Novel Anticancer

Alfonso Dueñas-González and Mandy Juárez-Rodríguez

Abstract

Drug repositioning is a alternative strategy to discover and develop anticancer drugs based on identification of new mechanisms of actions and indications for existing compounds. Ivermectin belongs to the avermectin group of compounds, a series of 16-membered macrocyclic lactone moieties discovered in 1967 and FDA-approved for human use since 1987. Ivermectin has since been used by millions of people worldwide, and have demonstrated a wide margin of clinical safety. Here we summarize the *in vitro* and *in vivo* evidence demonstrating ivermectin's potential as a multitargeting anticancer drug that exerts antitumor effects against different tumor types. Notably, the *in vitro* and *in vivo* antitumor activities of ivermectin are achieved at concentrations that can be clinically achieved based on human pharmacokinetic studies done in the clinical studies. Moreover, repurposed ivermectin safety has been well established recently in clinical studies against COVID-19. Consequently, we believe that ivermectin is an excellent potential candidate drug that can be repurposed for cancer and deserves rigorous evaluation against a variety of cancers in well-designed clinical trials.

Keywords: Drug repurposing, ivermectin, cancer

1. Introduction

Avermectins are a complex of 16-membered macrocyclic lactones produced from soil fermentation of the actinomycete *S. avermitilis* [1, 2]. There exist eight avermectin compounds (A1a, A1b, A2a, A2b, B1a, B1b, B2a, and B2b), of which ivermectin is the most commonly employed due to its semi-synthetic mixture (80% B1a and 20% B1b), and its potent antiparasitic activity as well as its safety [3]. The family of compounds from which Ivermectin is derived was discovered by Nobel laureates Satoshi Omura and William Campbell in the 1970s. The chemical is effective against a wide number of parasites and arthropods - pinworms, mites, lice, heartworms and fleas in dogs, parasitic worms in pasture animals by disrupting the fluid exchange through the insect's cell membrane, and in the past 40 years, ivermectin has been used extensively for agriculture and veterinary purposes [4–7].

The success of ivermectin treatment as antiparasitic is due to its high affinity for the glutamate-gated chloride channels (Glu-Cl) present in parasite cells but absent in vertebrates. The ivermectin-channel-interaction prevents channel closure,

leading to plasma membrane hyperpolarization, paralyzing the target parasite's pharyngeal and somatic muscles, triggering its death [2]. In addition to activating the Glu-Cl parasites channels, ivermectin acts as a dose-dependent positive allosteric regulator of several vertebrate ligand-gated channels, including the γ-aminobutyric acid type-A receptor (GABA receptor), glycine receptor, neuronal α7-nicotinic receptor, and purinergic P2X4 receptor. The effects of ivermectin over these receptors include the potentiation of agonist-induced currents at low concentrations and channel opening at higher concentrations [8]. However, GABA-sensitive neurons are protected by the blood–brain barrier within the central nervous system, protecting vertebrates against the potentially harmful effects of Ivermectin [3, 6].

2. Drug repurposing in cancer therapy

Effective, safe, and affordable cancer drugs are highly needed to reduce cancer mortality. The field of drug repurposing emerged in the early 1990s as an alternative to the conventional drug discovery model. This model entails targeting discovery and validation, lead identification by high-throughput screening, and lead optimization by medicinal chemistry. Drug repurposing surged to overcome the pharmaceutical industry's limited productivity regarding the number of approved drugs concerning the long time and huge money required to develop a drug. Classical drug discovery requires an average of 15 years of research, whereas drug development by repurposing is portended to be cheaper, faster, and safer. The significant advantage of drug repurposing is that the pharmacokinetics, pharmacodynamics, and toxicity profiles of drugs are, in general, well known; thus, its rapid translation into phase II and III clinical trials is feasible [9]. Among the different drugs currently studied under the focus of therapeutic repositioning, ivermectin is very promising. It has been shown to have antitumor effects *in vitro and in vivo* (**Figure 1**).

3. Antitumor effects of Ivermectin-mechanisms of action and *in vitro* data

Ivermectin has demonstrated antitumor effects in different types of cancers. Among mechanisms of action reported, ivermectin interacts and affects the function of 1) mitochondrial I complex, the multidrug resistance protein (MDR), 2) RNA helicases, 3) the WNT-TCF pathway, 4) chloride channel receptor, 5) immunogenic cell death via ATP- and HMGB1, 6) PAK-1, 7,8) epigenetic signature and sel-renewal of stem cells [10]. Preclinical testing have demonstrated inhibition of cell growth, induction of apoptosis in different cancer cell lines and antitumor effects in murine models (**Figure 1**) [11–19]. The *in vitro* antitumor effects are observed at a median concentration of 5 μM (0.01–100 μM), which is clinically attainable according to the pharmacokinetic data in humans shown in **Table 1**. We present a review of the laboratory results of ivermectin on various cancer cell lines below.

3.1 Ovarian cancer

Ivermectin blocks the oncogenic kinase PAK1 in human ovarian cancer and in NF2-deficient Schwannoma cell lines to suppress their PAK1-dependent growth in cell culture at a half maximal inhibitory concentration (IC50) between 5 and

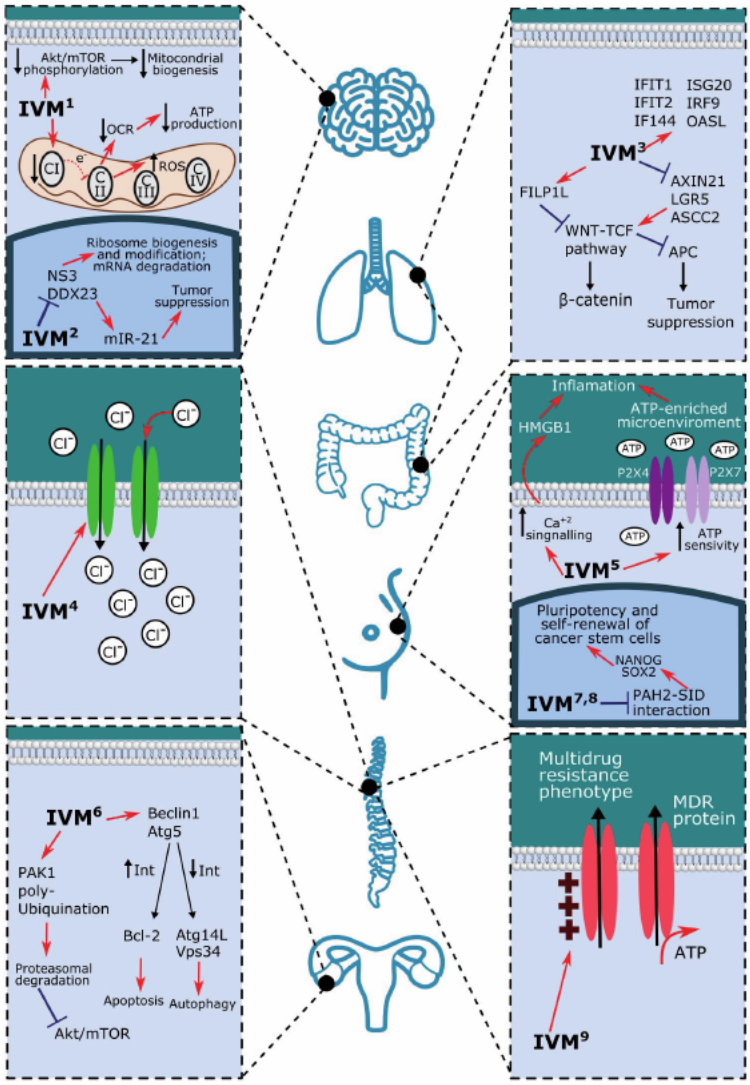

Figure 1.
Cancer targets of ivermectin. 1. Decreasing the function of the mitochondrial complex I, Ivermectin, limits the electronic movement in the oxidative phosphorylation pathway that stimulates oxygen consumption rate to generate ATP for the cell. Low ATP levels are related to a failure in the P-glycoprotein pump to extrude chemotherapy drugs. Concomitantly there is a reduction in the phosphorylation levels of Akt, impacting the mitochondrial biogenesis process. Furthermore, alterations in the mitochondrial machinery are related to increased levels of reactive oxygen species that damage DNA. 2. Ivermectin limits the function of the RNA helicases NS3 and DDX23, both of which are related to ribosome biogenesis and post-transcriptional modifications, as well as with mRNA degradation. DDX23 acts as a promoter of miR-21, which is a well-recognized stimulator of tumor progression. 3. The WNT-TCF pathway, involved in cancer progression and metastases, is inhibited by Ivermectin. Indeed, this compound represses AXIN2, LGR5, and ASCL2, all of them WNT-TCF targets. At the same time, it promotes the repressor of the WNT signaling FILIP1L. Both effects inhibit the ability of WNT-TCF to downregulate the tumor suppressor APC and limit the translocation of β–catenin to the nucleus for epithelial to mesenchymal transition in metastatic events. 4. Ivermectin acts as an ionophore by the up-regulation of chloride channels to generate apoptosis and osmotic cell death. 5. Ivermectin induces immunogenic cell death by stimulating an ATP- and HMGB1-enriched microenvironment, which promotes inflammation. This drug also increases ATP sensitivity and calcium signals in P2X membranal receptors, particularly P2X4 and P2X7, to induce ATP-dependent immune responses. 6. Ivermectin promotes the poly-ubiquitination of the kinase PAK1, which directs it to degradation in the proteasome. Defective PAK1, in turn, inhibits the Akt/mTOR pathway. At the same time, Ivermectin stimulates the expression of Beclin1 and Atg5, both related to induction of autophagy. Particularly, Beclin1 increases the expression of the positive autophagy regulators Atg14L and Vps34 and reduces the negative regulator of apoptosis Bcl-2. Together, this generates autophagy and apoptosis. 7,8. Ivermectin modifies the epigenetic signature and the self-renewal activity in the malignant cell due to its ability to mimic the SIN3-interaction that binds to the PAH2 motif of the ca.

Illness/Adverse effects	Mild	Intermediate	Severe
Onchocerciasis	Myalgia, skin eruptions, joints swelling, limbs or face, itching, fever and cold	Skin pain and edema, arthralgia, bone pain, severe dizziness, high fever, dyspnea, and hypotension	NA
Filariasis	Headache and nausea	NA	Encephalopathy
Scabies	Nausea	Severe headache, abdominal pain, and tachycardia	NA

Table 1.
Adverse effects caused by Ivermectin.

20 μM [14]. PAK1 is involved in various signaling pathways that play an essential role in cytoskeletal dynamics, cell adhesion, migration, proliferation, apoptosis, and mitosis. It is required for the growth of approximately 70% of neoplasms [20]. Additionally, cancer stem-like cells derived from SKOV-3 cell line treated with 5 μM ivermectin showed a significant decrease in cell viability and clonogenic capacity. Also, the expression levels of Nanog, Sox2, and Oct4 are reduced after treatment with ivermectin 5 μM [11].

3.2 Breast cancer

Ivermectin inhibits the ATK/mTOR pathway in breast cancer cell lines by promoting ubiquitination of PAK1. Ivermectin disrupts the binding of PAK1 protein with AKT, and in turn hinders the phosphorylation and activation of AKT; resulting in AKT/mTOR pathway inactivation. These effects of ivermectin are observed at concentrations above 10 μM [15]. Additionally, ivermectin preferentially inhibits the viability of cancer stem-like cells enriched populations (CD44+/ CD24−) in the range of 0.2–8 μM via reducing the expression of maintenance of the pluripotency and self-renewal markers Nanog, Oct4, and Sox2 at both mRNA and protein levels [11]. Separately, a study demonstrated that 1 μM ivermectin treatment inhibits the function of SIN3 [16], which is part of a complex that positively regulates Nanog and Sox2, leading to a decrease in mammospheres number [21]. Furthermore, ivermectin was reported to induce E-cadherin and Estrogen Receptor 1 expression and the restoration of tamoxifen sensitivity in a triple-negative breast cancer model. According to these observations, ivermectin has potential antitumor effects in triple-negative breast cancer [16]. Another study demonstrated a synergy between ivermectin with docetaxel or cyclophosphamide in estrogen receptor-negative breast cancer cells and a synergistic effect with tamoxifen in estrogen receptor-positive breast cancer cell lines [22].

3.3 Liver cancer

In human combined hepatocellular-cholangiocarcinomas and intrahepatic cholangiocarcinomas (cHC-CCs and ICCs), there is robust YAP1 activation. YAP1 is a transcriptional regulator of genes involved in cell proliferation and suppression of apoptotic genes, and itis inhibited in the Hippo signaling pathway which allows tumor suppression. Nuclear translocation of YAP1/TAZ also increases transcription of TGF-βs [23]. Thus, it is possible that coordinated targeting of YAP1/TAZ and TGF-β signaling may be a treatment for cHC-CCs and ICCs displaying dysregulated Hippo signaling and meanwhile drug screening revealed ivermectin to inhibit YAP1 activation [23].

3.4 Cervical cancer

Ivermectin inhibits the viability of HeLa cells and induces a G1/S cell cycle arrest leading to apoptosis and morphological changes of DNA fragmentation and chromatin condensation of such cells. Additionally, ivermectin can significantly increase intracellular ROS content and inhibit the migration of HeLa cells [24].

3.5 Glioblastoma

Ivermectin inhibits the growth of glioma cells by inducing cell cycle arrest and apoptosis in vitro and in vivo [25]. Specifically, in glioblastoma and brain endothelial cells, ivermectin has been reported to induce mitochondrial dysfunction. It inhibits cell growth and colony formation and blocks the enzymatic activity of the respiratory chain complex I, thereby decreases mitochondrial respiration, membrane potential, and ATP levels while increasing the generation of superoxides that in turn induces cell death by caspase-dependent apoptosis. Additionally, ivermectin also inhibits angiogenesis at concentrations above 5 μM [12].

3.6 Leukemia and prostate cancer

The treatment of OCI-AML2 cells with ivermectin increased the concentration of intracellular chloride ions, leading to hyperpolarization of the plasma and mitochondrial membranes and ROS production [18]. In contrast, DU145 and PPC-1 cells and primary normal hematopoietic cells that were resistant to ivermectin did not demonstrate changes in their plasma membrane potential when treated with up to 6 μM ivermectin. Moreover, the *in vitro* antitumor effect of ivermectin on various cancer cell lines at a concentration of 5 μM showed that DU145 is only minimally reduced in viability and clonogenic capacity, but when it is treated in combination with docetaxel cells demonstrated strong inhibition [22]. In myeloid leukemia cells ivermectin strongly synergizes with daunorubicin and cytarabine [18].

3.7 Colon and lung cancer

The WNT/TCF signaling pathway is constitutively active in many tumors and it regulates genes for cell growth and proliferation. Ivermectin can inhibit the WNT-TCF signaling pathway by decreasing cyclin D1, which is a direct target in this pathway and ivermectin also affects the phosphorylation of β-catenin, which leads to inhibition of proliferation and increased apoptosis in lung and colon tumor cells at concentrations above 5 μM [13].

4. Antitumor effects of ivermectin-animal data

In a wide-range of pre-clinical studies, rodent models of human xenografts of glioblastoma, leukemia, breast and colon carcinomas, as well as a variety of murine cell lines in syngeneic models have consistently shown ivermectin to possess robust antitumor effect at a median dose of 5 mg/Kg [12, 13, 15, 17, 18]. We present a review of some results of anticancer studies of ivermectin in animal below.

4.1 Glioblastoma

Two independent glioblastoma xenograft SCID mice models were established by subcutaneous injection of U87 or T98G cells, and the rodents were subsequently

treated with intraperitoneal ivermectin at 40 mg/Kg. The treated mice had demonstrated significant tumor growth inhibition but maintained normal behavior and retained their weight [12]. A separate study using 3 mg/Kg of ivermectin showed a 50% decrease in tumor size and there was near complete regression of tumors at 10 mg/Kg. Ki67 staining also confirmed that glioma cell proliferation was decreased in ivermectin-treated animals compared to controls [17].

4.2 Colon and lung cancers

Melotti et al. used H358 human metastatic lung bronchioalveolar carcinoma cells and DLD1 colorectal adenocarcinoma cells to test the antitumor effects of ivermectin. The animals received intraperitoneal injections of cyclodextrin-conjugated ivermectin daily at 10 mg/kg after tumor establishment. Subsequently, it was found that tumors responded to ivermectin with a near 50% reduction of growth and a repressed lung cancer WNT-TCF signature and enhanced p21 levels [13].

4.3 Breast cancer

Ivermectin was evaluated in an orthotopic breast cancer model with human MDA-MB-231 cells subcutaneously injected in the mammary fat pad of NOD-SCID mice. Xenografts treated with ivermectin grew at a slower rate than those of the control group, and the size and weight of control tumors were macroscopically larger than that of ivermectin-treated tumors [15]. Another study tested JC murine breast cancer cells in Balb/c mice treated with a dose of 3 mg/Kg of ivermectin. Treatment reduced tumor size more than 60% with no changes in weight or behavior of the study animals when compared with controls [22]. Recently it was demonstrated the ivermectin at a dose of 5 mg/Kg induces immunogenic cell death hallmarks with large numbers of intratumoral CDA4+ and CD8+ T cells in a 4 T1 murine tumor model. Thus, ivermectin turns cold tumors into hot ones which allows for marked synergy with check point inhibitor nivolumab, leading to major antitumor effects and most importantly, protective immunity [26].

4.4 Leukemia

Human leukemia (OCI-AML2 and K562) and murine leukemia (MDAY-D2) cells were injected subcutaneously into NOD/SCID mice which were subsequently treated with 3 mg/Kg (human equivalent dose of 0.240 mg/Kg) of ivermectin or control in water via oral gavage. Upon follow-up, the treated mice had up to 70% decrease in their tumor burden without any gross sign of organ toxicity, and treatment led to increased apoptosis in OCI-AML2 xenografts [18]. It must be remarked that most of the in vivo studies to evaluate the antitumor effects of ivermectin dose ranging from 3 to 10 mg/Kg. These mice doses translate into human to 0.240 to 0.810 mg/Kg which are clinically attainable [27].

5. Clinical experience with ivermectin

As mentioned above, there has been extensive clinical use of ivermectin as an antiparasitic, and the drug has been repurposed for use against other pathogens and nonparasitic conditions in humans. However, despite considerable preclinical evidence of antitumor effects of ivermectin, it is curious that no clinical studies of ivermectin against cancer have been reported nor clinical trials launched. However, there is a case report on three children with refractory and heavily pretreated acute myeloblastyic

leukemia. In the three cases, ivermectin was at 1 mg/Kg either alone or in combination with Ara-C. Two of them had clinical improvement with durable stable disease in one, a and complete hematological response the second. The third one receiving ivermectin alone had no response. Though anecdotic, these data demonstrate that ivermectin can be safely administered at dosis five times higher the recommended dose of 0.200 mg/Kg, and that can show efficacy combined with cytotoxics [28].

Here, we briefly review the clinical experience with ivermectin as an antiparasitic as well as in other repurposed indications, with special attention to its toxicities and safety and its clinical pharmacology, the data of which can be a basis for future clinical trials of ivermectin against cancer.

5.1 Ivermectin use as anti-parasitic

Because of its broad spectrum applicability, ivermectin can be applied to treat onchocerciasis, lymphatic filariasis, strongyloidiasis, ascariasis, scabiasis, and enterobiasis. Since its discovery, ivermectin has been administered to millions of patients with the above parasitic infections around the world. Mild adverse effects of oral ivermectin therapy against certain parasites are common; many of them appear within 24-48 hours of the onset of therapy and are related to ivermectin dose as well as the microfilariae load in the skin in case of filiariasis [29, 30]. Some of these adverse effects include myalgia, skin rashes, joints swelling, limbs or face itching, fever, and chills. These effects are usually transient and do not require treatment [31, 32]. Moderate to severe effects are less common and may include skin edema with the presence of pain, arthralgia, severe dizziness, high fever, dyspnea, and hypotension (Mazzotti's Reaction). It is known that such reaction is not related to the administration of Ivermectin but with the parasite amount present in the host [30, 31]. In addition to Mazzotti's reaction, there have been cases of severe encephalopathy that can be fatal in patients with onchocerciasis and filariasis after treatment with ivermectin. The symptoms of encephalopathy include altered mental status, incontinence, and difficulty standing or walking 48 hours after ivermectin treatment [32, 33]. This effect is again probably due to the obstruction of the cerebral microcirculation due to the accumulation of paralyzed or killed parasites and not by ivermectin itself [34, 35]. Also, toxic effects have been linked to ivermectin's interaction with P-glycoprotein [8]. The absence of P-glycoprotein determines the accumulation of Ivermectin in the brain of transgenic mice who do not express it and dogs with impaired P-glycoprotein function (commonly a 4 base-pair deletion of the MDR-1 gene that produces a stop codon) have increased neurotoxicity to ivermectin [36]. **Table 2** summarizes ivermectin's adverse effects. The dose and schedules vary but human doses are standardized for approved indications within the range of 0.15 to 0.4 mg/Kg. For onchocerciasis, the recommended dose is 0.15 mg/Kg once every 12 months, though patients with heavy ocular infection may require retreatment every 3 or 6 months. Filariasis usually requires a single dose of

Group	Dose (mg/kg)	Drug delivery	Cmax (ng/mL)	Tmax (h)	AUC μg/h/mL
Onchocercosis patients	0.1–0.2	Oral	52.0	5.2	2.852
Healthy volunteers	0.35–0.6	Oral	87.0	4.2	1.444
Healthy volunteers	0.7–1.1	Oral	165.2	3.6	2.099
Healthy volunteers	1.4–2.0	Oral	247.8	4.2	4.547

Table 2.
Pharmacokinetic data of Ivermectin in humans infected with parasites and in healthy volunteers.

0.4 mg/Kg. In strongyloidiasis, a single dose of 0.2 mg/Kg is recommended; however, in immunocompromised (including HIV) patients, the treatment may require repeated administration (i.e. every two weeks) and continued suppressive therapy (i.e. once a month). A single dose of 0.2 mg/Kg is also used to treat ascariasis, while the same dose repeated once at two weeks is recommended for scabiasis [37].

Recently, there has been a growing interest in newer anti-parasitic indications of ivermectin such as against soil-transmitted helminths and malaria, hence doses above 0.4 mg/Kg are being evaluated for achieving higher plasma levels [38, 39].

An example is a pharmacokinetic trial using 18 mg ivermectin tablets in 54 healthy adult volunteers to evaluate the safety of fixed regimens of 18 and 36 mg [40]. A meta-analysis to investigate the safety of higher doses of ivermectin identified four studies for inclusion, and found no differences in the number of individuals experiencing adverse events at higher doses. A descriptive analysis of these clinical trials for a variety of indications also showed no difference in the severity of the adverse events between standard (up to 0.4 mg/Kg) and higher doses of Ivermectin (0.4-0.7 mg/Kg; 0.6 mg/Kg, and 0.8 mg/Kg). Only one trial showed an increase in transient and mild to moderate subjective ocular events such as transitory blurring of vision, itching or pain of the eye, and dyschromatopsia in the higher-dose group in a trial to treat onchocerciasis. Meanwhile, severe adverse events described as life-threatening, was reported in only one out of the four studies with one case of anaphylaxis at the standard dose and another case of QTc prolongation likely due to drug-drug interaction in a higher-dose group [41]. The result of this small meta-analysis is suggestive of relatively safety of higher doses of ivermectin.

5.2 Ivermectin's potential as an anti-viral

Ivermectin exhibits anti-viral activity against viruses both *in vitro* and *in vivo*. The antiviral activity is thought to be related to tthe inhibition of nuclear translocation of viral proteins, facilitated by mammalian host importin also known as karyopherin α/β-1 heterodimerization [42]. It is partially upon this basis that ivermectin has been tested as a treatment in the current COVID-19 pandemic. A recent meta-analysis and systematic review involving 629 COVID-19 patients from 4 observational studies (3 with control arms and 1 without) found that adding ivermectin led to significant clinical improvement compared to control (OR=1.98, 95% CI: 1.11 - 3.53, p=0.02) [43]. however, the authors did caution on the interpretation of their analysis because the low quality of evidence, and it should be noted that one of the trials included in the analysis was subsequently retracted. Meanwhile, several randomized studies evaluating ivermectin against COVID-19 have recently been published. An Iranian trial demonstrated that a single 0.2 mg/Kg dose of ivermectin was well-tolerated in symptomatic COVID-19 patients, and dyspnea, cough and lymphopenia associated with COVID-19 were significantly improved [44]. In two other randomized trials, the time to viral clearance was statistically reduced. The doses and schedules in these two trials were ivermectin at a fixed 12 mg daily for 5 days [45] and ivermectin at 0.1, 0.2, and 0.4 mg/Kg once at admission [46]. These were underpowered trials so that further evidence is still required to confirm the clinical usefulness of ivermectin under various COVID-19 clinical scenarios.

5.3 Other uses of ivermectin

Ivermectin possesses possible agonistic bioactivity against the γ-aminobutyric acid (GABA) receptor [47] and it was upon this premise that it was used in a patient with severe spasticity caused by spinal cord damage at a dose of 1.6 mg/

Kg subcutaneously twice a week for 12 weeks. The patients had decreased spasm scores, suggesting that ivermectin may reduce spasticity in the spine without adverse effects at this high dose [48].

6. Pharmacokinetics and dose considerations for ivermectin as cancer therapy

Due to its relatively long history of extensive use, the pharmacokinetics of ivermectin has been well studied. The oral route is the only approved for ivermectin administration in humans although it can be given subcutaneously and the intravenous route of administration has also been investigated. Ivermectin is a fat-soluble compound and reaches a peak concentration 4-5 hours after oral administration, and it has a half-life of approximately 19 hours. After administration, it is subsequently extensively metabolized in human liver microsomes by cytochrome P-4503A4, converting the drug to at least ten metabolites, most of them hydroxylated and demethylated derivatives. Its excretion is mainly by the fecal route, and only 1% is excreted in the urine [49]. In healthy individuals and patients infected with onchocerciasis treated with a dose of 0.150 mg /Kg of Ivermectin, significant variability in pharmacokinetic parameters such as absorption, distribution, metabolism, and excretion is not observed [49].

The therapeutic dose for ivermectin as an anti-parasitic compound for human use is is between 0.1 and 0.4 mg/ Kg [4–7], resulting in an AUC of 1,444 μg/h/mL. This drug exposure, which translates to a plasma concentration of 1.65 μM, is however less than concentrations of 5 μM or greater that has been found necessary to inhibit tumor cells *in vitro* In a phase I pharmacokinetic study done in healthy volunteers, it was demonstrated that doses up to 2 mg/Kg which leads to an AUC of 4,547 μg/h/mL can translate into a plasma concentration of 5 μM [50], thus the recommended dose for cancer therapy should likely be 2 mg/kg or higher.

7. Discussion

Currently, various efforts to facilitate the discovery of drug repurposing candidates for cancer and a large number of drug candidates do exist [51]. As an example, the Repurposing Drugs in Oncology (ReDO) Project, which is initiated by a non-profit international collaboration of researchers, clinicians, and cancer patient advocates whose goal is to find efficacious, minimally toxic, and affordable cancer treatments identified a total of 268 drugs that matched the following two criteria: i) the drug is licensed for non-cancer indications in at least one country in the world, and ii) the drug is the subject of one or more peer-reviewed publications showing a specific anticancer effect based on *in vitro*, *in vivo*, or clinical research in one or more malignancies. According to these criteria, ivermectin can be a potential repurposing candidate for cancer. Ivermectin has extensive preclinical *in vitro* and *in vivo* anticancer data and is thus an ideal candidate for clinical trials. An especially promising feature with ivermectin is that its anti-cancer concentration *in vitro* should be attainable clinically, inexpensively, and without undue toxicity.

8. Conclusion

Ivermectin has been administered to millions of patients as an anti-parasitic drug exhibiting a wide margin of clinical safety. There exists a large body of *in vitro*

and *in vivo* evidence demonstrating ivermectin's anti-tumor potential, and ivermectin's anti-tumor efficacy can be demonstrated at concentrations that are clinically attainable based on clinical pharmacokinetics. We thus propose that ivermectin be considered urgently for clinical trials either as a single agent or in combination with existing antineoplastics for cancer.

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Progesterone and Glucocorticoid Receptor Modulator Mifepristone (RU-486) as Treatment for Advanced Cancers

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Abstract

The fetal placental unit has paternal proteins which would normally result in immune rejection of fetus. Thus, to allow growth to 266 days, the mother must develop immunosuppressive proteins, cytokines, etc. to allow progression to a full-term baby. One of these essential immunomodulatory proteins is called the progesterone induced blocking factor (PIBF). Probably, the mechanism involved allowing the progesterone receptor antagonist mifepristone to cause termination of a pregnancy is by blocking the PIBF protein. There is good evidence that cancerous tumors borrow some of the same mechanisms as the fetus to escape immune surveillance, including the PIBF protein. Research data suggest that this protein is made and excreted by embryonic cells, mesenchymal cells, and trophoblast cells of the fetal placental unit to block the killing effect of natural killer cells and T-cells in the fetal microenvironment. Cancer cells do the same. Indeed, there is good evidence that mifepristone, a drug approved for pregnancy termination, can significantly improve length and quality of life in patients with various advanced cancers.

Keywords: progesterone induced blocking factor, metastatic cancer, progesterone receptor antagonists, natural killer cells, membrane progesterone receptors

1. Introduction

A certain minimal level of progesterone must be maintained from ovulation until delivery to allow the birth of a full-term live baby [1]. Progesterone (P), acting in conjunction with the P receptor, causes the production of a large number of various molecules needed for the development of an appropriate secretory endometrium to allow attachment of the blastocyst to the endometrium and adequate invasion to the proper depth of the fetal placental unit [1].

Some of the molecules induced are also needed to suppress rejection of the fetal semi-allograft. One of these immunomodulatory proteins has been termed the progesterone induced blocking factor (PIBF) [2]. There is evidence that PIBF is one of the most important immunomodulatory factors produced during pregnancy to inhibit immune rejection of the fetal semi-allograft [3, 4].

Progesterone-induced blocking factor is an immunomodulatory protein that can suppress or block various aspects of the immune system, especially, but not

limited to, natural killer (NK) cells [5, 6]. The blocking effect on cellular immunity, especially NK cell cytolytic activity, may be related, at least in part, to a shift from thymic helper (TH)-1 to TH2 cytokine dominance [7]. One mechanism by which PIBF can suppress NK cell cytolytic activity is by inhibiting degranulation of perforin granules, one mechanism used by NK cells to kill other cells [8].

The "parent" form has a molecular mass of 90 kDa and is localized in the centrosome [9]. Various splice variants of this nuclear protein lead to smaller intracytoplasmic molecules that have immunosuppressive activity [9]. The actual full-length protein contains 757 amino acids, and the 48 kDa N terminal part is biologically active [10]. The PIBF gene has been identified on chromosome 13 in the vicinity of breast cancer 1 (BRCA1) or BRCA2 or p53 [11, 12].

Progesterone-induced blocking factor rises precipitously in the serum after exposure to P (even in males injected with progesterone) and the source seems to be circulating gamma/delta T cells [2]. However, it seems that the main source of PIBF that allows the early feta-placental to escape immune surveillance are actually cells of the fetal placental unit namely embryonic cells, mesenchymal cells, and trophoblast cells [1, 9].

In 2001, Check et al. hypothesized that it is likely that cancer cells might "borrow" some of the same mechanisms to escape immune surveillance as the fetal-placental unit [13]. Based on their previous research with the PIBF protein, they considered that, whereas treatment for infertility or recurrent miscarriage should be aimed at increasing the production of the PIBF protein, theoretical treatment for cancer could be therapy aimed at suppressing the PIBF protein [13].

Support for this concept was provided by Lachman et al., who showed that many different types of cancer cells express this PIBF protein [9]. Though one may think that highly proliferating cancer cells may be the ones that have the classic nuclear progesterone present, the study by Lachman et al., found many of the cancers associated with PIBF were not known to be positive for the nuclear P receptor [9].

Based on this hypothesis, it was considered that a P receptor antagonist/modulator should cause suppression of PIBF production in rapidly growing cancer cells which could overcome the theoretical block of immune function of cellular immune cells in the tumor microenvironment.

Mifepristone was the first P receptor antagonist developed [14]. It was a derivative of the synthetic progestin norethindrone [14]. It was purposely developed to be an abortifacient to alter the endometrium and cause decidual necrosis and cause the trophoblast to separate from the decidua [14–16]. Mifepristone sensitizes the pregnant uterus and cervix to endogenous and exogenous prostaglandins increasing uterine contractility and helps to induce cervical softening [14–16].

Over the years other benefits of mifepristone, related to its anti-progesterone effect, have been developed, including treating uterine leiomyomata and endometriosis [17]. The anti-abortifacient drug comes in 200 mg tablets. Since mifepristone in higher dosages blocks the glucocorticoid receptor, it has been approved as a 300 mg tablet to treat Cushing's syndrome [18].

Thus, we set up a study to determine if we could detect PIBF in various leukemia cell lines, and, if so, determine if adding mifepristone to the medium could reduce PIBF secretion. To do so we collaborated with Dr. Srivastava from the Roswell Park Cancer Institute, who for many years studied protein production by leukemia cell lines. Twenty-nine cell lines of diverse lineage were all found to express messenger (m) RNA for PIBF [19]. In fact, there was more mRNA dedicated to the production of the PIBF protein, by far, than any mRNA for any other protein previously studied in these leukemia cell lines [19]. Ten cell lines positive for mRNA for PIBF were tested for the PIBF protein using a much less sensitive assay for PIBF than is presently available. Four tested positive for the PIBF protein. Addition of progesterone to the

media of the cell lines up-regulated mRNA for PIBF and also the PIBF protein [19]. In contrast, the addition of mifepristone to the media down-regulated both mRNA for PIBF and the 35 kDa PIBF intracytoplasmic splice variant protein (similar in size to the PIBF splice variant in fetal-placental cells) [19].

Subsequently studies using other cancer cell lines supported the conclusions from the leukemia cell line studies. Kyurkchiev et al. found that glioblastoma multiforme also express the intracytoplasmic PIBF protein, but in this case the splice variant measured 57 kDa [20]. Gonzalez-Arenas et al. found, similar to the aforementioned leukemia cell line studies, adding P to the media up-regulates the 57 kDa intracytoplasmic splice variant of PIBF in glioblastoma multiforme cell lines [21]. Interestingly, in addition they added PIBF protein to the media and found that PIBF increased the number of U87 cancer cells on days 4 and 5 of treatment. This suggests that PIBF promotes proliferation of human glioblastoma cancer cells independent of an intact immune system, which would require a whole intact animal or human [21].

Mifepristone has been also found to inhibit the growth of cell lines or murine tumor transplantation from endometrial cancer, breast cancer, prostate cancer, gastric cancer, ovarian cancer, and lung cancer [22–27].

Goyeneche's group published some interesting findings concerning mifepristone and ovarian cancer cell lines. They have found that mifepristone inhibits ovarian cancer cell growth in vitro and in vivo [28]. They have published several studies showing the benefit of the combination of mifepristone and chemotherapy with cisplatin therapy or cisplatin-paclitaxel treatment of ovarian cell lines [29–31].

Based on these cell line studies, more support was provided that cancer cells may borrow some of the same escape mechanisms as the fetal-maternal unit to escape immune surveillance. Thus, therapy aimed to suppress these immune factors could lead to novel effective anticancer therapies [32]. Dr. Szekeres-Bartho, another pioneer in determining that the immunomodulatory protein, PIBF, plays a major role in allowing the fetus to avoid immune surveillance, in 2010 wrote a treatise entitled "PIBF: the double-edged sword. Pregnancy and tumor" [33].

In an opinion entitled "Pregnancy is a model for tumors, not transplantation," the renowned immunologist Kenneth Beaman, and his group, in 2016, stated "Nearly 65 years have passed since Peter Medawar posed the following question: "How does the pregnant mother contrive to nourish within itself for many weeks or months, a fetus that is an antigenic foreign body." Now, understanding of reproductive immunology has demonstrated that the HLA antigens in the placenta are non-classical and do not induce rejection. In the placenta and in tumors, 50% or more of the cells are cells of the immune system and were once thought to be primed and ready for killing tumors or "the fetal transplant" but these cells are not potential killers but abet the growth of either the tumor or the placenta. By examining the similarities of the placenta's and tumor's immune cells, novel mechanisms to cause tumors to be eliminated can be designed. Thus, 15 years later, the concept we published in 2001 is starting to be accepted by top immunologists in the field [34]. Though Beaman et al. do not refer at all to the PIBF protein, I recommend an article in gynecologic oncology to those readers wanting further knowledge into the immune similarities between pregnancy and cancer to open the door for other novel treatments of malignant tumors other than blocking the progesterone receptor [35].

2. Animal studies with mifepristone in cancers that are, and are not, known to Be associated with the classic P nuclear receptor

In humans, the progesterone receptor (PR) is expressed in prostate stroma. Reduced PR expression in cancer-associated stroma can be conducive to a tumor

microenvironment favorable for cancer cell invasion and tumor metastases [36]. Thus, if the presence of the PR somehow inhibits tumor invasion and metastases, treating with a PR antagonist may worsen the condition.

However, it may be that the loss of the PR receptor merely suggests a higher percentage of more aggressive cells, and thus, mifepristone, by suppressing PIBF, may inhibit prostate cancer proliferation. Indeed, gavaging mice with spontaneous prostate cancer with mifepristone (which on a weight basis was equivalent to 200 mg daily in humans) improved longevity of survival and body condition scores compared to placebo gavaged C57BL/6 mice [37].

Controlled studies were also performed in mice where there was no knowledge of the presence of the classic nuclear PR. Beneficial effect on longevity and quality of life (body conditioning score) were observed in 129 Pd/J mice with a strong predisposition for testicular cancer, in aldo-keto reductase/J mice with spontaneous lymphocytic leukemia and A/J mice with spontaneous lung cancer [37–39]. As an example, in A/J mice with spontaneous lung cancer, 67.4% treated with mifepristone survived 1 year vs. 27% of the controls [39]. Even more important, there were 66.7% of mice gavaged with the equivalent of 200 mg/day in humans with mifepristone who had no sick days (body conditioning score less than 4) vs. zero % for controls [39]. These murine carcinoma studies supported the concept that the benefit of mifepristone is not merely for cancers positive for the classic nuclear PR. If the mechanism of improvement did operate through the PIBF mechanism, the presence of the classic nuclear PR is not needed for production of PIBF expression by the tumor cells.

3. Case reports

Based on cell line studies and controlled animal studies, we wanted to determine if the mifepristone could provide increased longevity and/or improved quality of life in human patients with advanced cancer. Unfortunately, though physicians generally have the right to use drugs off-label, there was a restriction for mifepristone. This was not related to risk of the drug, but related to appeasing antiabortion groups who feared that the drug could find easy use to cause abortions. Thus, to use mifepristone as an anticancer drug, one needs to obtain from the Food and Drug Administration a compassionate use investigational new drug (IND) approval to use mifepristone to treat cancer.

3.1 Case 1

The first patient we treated with oral daily mifepristone 200 mg/day was a 46-year-old woman diagnosed with a rare thymic epithelial cell cancer. Over a one-year period following initial surgery and radiotherapy more cancerous lesions developed in the lung. There was no standard chemotherapy, but she was approved for experimental octreotide. However, the cancer still progressed. After starting mifepristone 200 mg/daily, though, her lung and mediastinum lesions did not regress, they remained stable. Clinically, she was feeling much better in that she had much less shortness of breath, much less cough and, marked improvement in fatigue. This clinical improvement persisted for over 2 years. Her oncologist decided that since the lesions were stable, this could be the opportunity to attempt a “cure” by a second course of radiotherapy to the mediastinum. She developed pulmonary fibrosis from this second course of radiotherapy. According to the thymic Cancer Carcinoma Society, she had survived the second longest time of any patient with this type of cancer [40]. Now, with more clinical experience, she would have

been advised against more radiotherapy and just continue the mifepristone. Most metastatic cancers will not be "cured." The end point of treatment with mifepristone should be quality of life and increased longevity. This first case of our series of anecdotal cases treated with mifepristone first started treatment in 2004. It is important to note that thyroid epithelial cell cancer is not known to be associated with the classic nuclear P receptor.

3.2 Case 2

The second case of advanced cancer that we obtained a compassionate use IND to treat was a 61-year-old woman with a 6.5 cm invasive moderately differentiated adenocarcinoma of the transverse colon with extensive metastasis to the liver, peritoneum, ovary and uterus. She had marked ascites. The two largest liver metastases measured 3.1 × 1.3 cm and 2.3 × 1.9 cm. She was advised by her oncologist that even with chemotherapy she would only have a 15% chance of living 6 months.

After 1 year of mifepristone therapy 200 mg orally per day her carcinoembryonic antigen level had dropped all the way down to 1.6 ng/mL. After 18 months, there had not been any growth of her metastatic lesions nor did any new ones appear. She had no pain, no vomiting, and she stated her energy was great.

A CT-scan at 22 months showed some growth of the lesions. Nevertheless, she was pain free with good energy even at 27 months when ascites began to return (it had completely disappeared). She was still ambulatory at 30 months when she died.

Several years later talking to her sister we found out that at 18 months, to save money, she started taking the mifepristone every other day. Thus, this case helps to establish that the daily dosage should not be less than 200 mg/day. The case also supports the concept that mifepristone can prolong life and provide palliation for cancers not known to be associated with the classic P nuclear receptor [41].

3.3 Case 3

Another 43-year-old woman with stage IV metastatic colon cancer, who had progressed despite standard chemotherapy, began single agent mifepristone therapy. Similar to the aforementioned case, there was a halt to cancer progression, her energy markedly improved, and she had great relief of pain. After 18 months some of her metastatic lesions began to grow. She assumed that this was the end of her remission, so she stopped the mifepristone, and decided to try a new experimental drug. She died 3 months later [40]. Based on subsequent clinical experience, we would have advised her that even though the lesions are starting to grow again, mifepristone will still prolong a high quality of life, and will prevent rapid spread, thus advising her not to stop mifepristone.

3.4 Case 4

An 83-year-old man with rapidly growing stage IV colon cancer with metastases to his lungs, liver, peritoneum, and lymph nodes showed no improvement to either capecitabine or cetuximab. He was so weak that he could not get out of bed. Within 2 weeks of 200 mg mifepristone tablets daily obtained with compassionate use his energy returned, and he was able to resume normal function and go to restaurants and other social events and completely take care of himself (ECOG 0 now). His appetite also returned, and he was pain free.

After 4 ½ months of therapy none of his previously rapidly growing metastatic lesions grew with the exception of 1 lung lesion that grew 0.3 cm. He had no side

effects from treatment. Though he had no kidney metastases, he had pre-existing marked renal impairment. He became uremic. His wife was deciding on dialysis or not when he died of a sudden myocardial infarction [41].

3.5 Case 5

Sometimes, instead of the mifepristone therapy causing stable disease, or changing the pattern from rapid progression to slow progression, the lesions may show marked regression. This is evidenced by a 45-year-old woman who had widely metastatic leiomyosarcoma despite previous treatment with total abdominal hysterectomy and bilateral oophorectomy, letrozole (the tumor was estrogen receptor positive), and gemcitabine/docetaxel, and resection of lung metastases [40].

She was started on mifepristone 200 mg/day orally. This caused an almost total remission, with disappearance of almost all lesions, and those remaining had shown marked decrease in size. After 6 months, some lesions began to appear, but they were still very small. Nevertheless, without experience with the nature of this drug, the oncologist opted to stop mifepristone and place her in an experimental trial. She died within 1 month from complications of this new drug [40].

3.6 Case 6

Another case of very rapidly growing advanced cancer showing complete remission following ingestion of 200 mg/day oral mifepristone was an 80-year-old woman with a history of chronic lymphocytic leukemia who developed sudden onset respiratory failure with a po_2 of 72 mmHg. Chest X-ray revealed many lung lesions with a radiographic diagnosis of probable advanced lung cancer with multiple metastatic lesions. Her serum sodium was 118 mmol/L. She refused a surgical diagnosis or chemotherapy based on the presumptive clinical diagnosis of small cell lung cancer with the syndrome of inappropriate anti-diuretic hormone (SIADH) and the bleak prognosis, even with chemotherapy [42].

She sought an alternative treatment and agreed to mifepristone therapy 200 mg orally daily. Within 1 month her po_2 returned to 99-100 mmHg without supplemental oxygen. Her serum sodium increased to normal at 145 mmol/L. Her CT-scans showed complete disappearance of all lung lesions even 5 years after initial diagnosis. There did remain, however, a ground glass appearance in the lungs. She died 5½ years later at the age of 85.5 from an acute myocardial infarction, not from lung cancer [42].

Interestingly, though we know that PIBF is secreted by leukemia cell lines and is suppressed by mifepristone, this woman's CLL slowly progressed while her rapidly growing presumed small cell lung cancer had a complete remission [19]. This could suggest that mifepristone acts better on rapidly growing cells than slowly growing cancers. Of course, it is possible that the mifepristone helped keep the CLL slow growing, but that could simply be related to the normal situation of slow progression with CLL even without treatment. It should be noted that lung cancer, whether small cell or non-small cell (which is still possibly the type of cancer this woman had though small cell was more likely because of the clinical picture) is not known to be associated with nuclear P receptors.

Many cancer therapies are ineffective for brain metastases or primary brain cancers because they cannot cross the blood-brain barrier. There is anecdotal evidence that mifepristone can cross the blood brain barrier and provide palliative benefits for primary brain cancer and brain metastases.

3.7 Case 7

A 43-year-old male with a 3-week history of severe protracted headaches was found to have a large glioblastoma multiforme grade IV that originated in the temporal lobe but involved also the frontal, parietal and temporal lobes and metastases to the spinal cord. Despite surgery, radio and chemotherapy, the tumor rapidly progressed. He was not considered a candidate for any other therapy. At the time of starting mifepristone therapy, he was paralyzed from the neck down and his hands were fixed in the clenched position. He slept most of the day, and when awake, was not able to carry out conversations [43].

Within 2 weeks of treatment with 200 mg oral mifepristone daily, he became much more alert and was able to carry out intelligent conversations. He was now able to open his clenched fists and move his hands. He continued treatment for 3 months and remained alert. However, his paralysis slowly progressed to the point where he was having trouble breathing and swallowing. The mifepristone was stopped, and he died 2 weeks later [43].

3.8 Case 8

Another case demonstrating that mifepristone can cross the blood brain barrier to thwart brain metastases from progressing is a case of a 68 year old male with stage IV metastatic non-small cell adenocarcinoma lung cancer with brain metastases who was referred by his oncologist for mifepristone therapy [44]. Based on the experimental data with efficacy of mifepristone inhibiting growth of cancer cell lines, the beneficial effect in controlled various murine carcinomas, and the anecdotal benefits in individual causes with various advanced cancers following single agent mifepristone therapy the FDA approved our investigator imitated study entitled "A phase II study of treatment with oral mifepristone as salvage therapy in patients who have failed two or more previous chemotherapy regimens" (www.clinicaltrials.gov).

He had no tumor markers that could provide him targeted therapy. His cancer progressed despite 3 rounds of multi-agent chemotherapy including carboplatin/avastin/docetaxel, pemetrexed, and gemcitabine. In October of 2015 he had a seizure and magnetic resonance imaging indicated a 1 cm right frontal lobe metastatic lesion. He received palliative stereotactic radiotherapy to the brain lesion which was completed in November 2015.

With deteriorating symptoms, for example, dyspnea on exertion and fatigue and with no other treatment options available (PD-L1 marker was negative and checkpoint inhibitors were not approved for PD-L1 negative patients at this time), he was referred for our FDA study.

In all previous cases, the 200 mg mifepristone tablets were obtained from Danco Inc. at a cost of about $500 per month. For the FDA approved investigator-initiated study, we decided to use mifepristone 300 mg tablets daily because the company Corcept, Inc. which manufactures the 300 mg tablet for treatment of Cushing's syndrome (though the dosage is generally much higher than 300 mg to block the glucocorticoid receptor) was willing to provide the drug free to approved patients.

His clinical symptoms improved significantly within 1 month of treatment with single agent oral mifepristone 300 mg daily. He was ECOG 1 at the start of therapy and after 1 month was ECOG zero. He remains ECOG zero after 4.8 years of treatment, and for the majority of visits, he answers his 43 questions on the quality of life evaluation as "not at all" (the best answer that could be given). There has been no evidence of growth of his previous brain metastases or any new lesions by MRI testing.

One additional important piece of information that his case provides. His metastatic lesions remained stable for 1.5 years. But after 1½ years, some lesions began to grow slowly. His oncologist, based on his experience with other anticancer agents, thought that once disease progression began, it usually accelerates rapidly. He thus suggested to the patient that he stop the mifepristone, and consider nivolumab or pembrolizumab, which had at this time been tried on some patients who were PD-L1 negative, or consider another biopsy to determine if a new tumor marker could be found that would allow targeted therapy. The patient feeling so good on mifepristone therapy and feeling so poorly on all of his previous chemotherapy regimens, opted to take our advice and continue on the mifepristone therapy. Now 3.5 years later and still feeling great, he is very satisfied with his decision not to stop mifepristone therapy [44].

This case exemplifies the mistakes, from lack of experience, that we alluded to in some of the previous case reports, that is, one should not stop the drug if there is the start of tumor progression. There is still a good chance the drug will provide continued extension of a good quality life. Naturally, if a new therapy is likely to be more effective than the mifepristone therapy, then it would make sense to try the new agent. But it makes no sense to try a completely new experimental drug with unknown side effects, as tried by some of the previous described cases. Furthermore, experience suggests that mifepristone inhibits metastases, but cessation of therapy results in rapid spread. This progression can be so rapid that it could be too late to resume mifepristone therapy if the new anticancer therapy is not working.

Therapy with mifepristone could be considered hormonal therapy, but because its hypothesized mechanism is that it removed a block (i.e., PIBF), and thus allows the cellular immune system (especially NK cells) to attack cancer cells, it could also be considered a form of immunotherapy. The question arises as to whether the drug would be effective in cancers positive for the programmed cell death protein ligand 1 (PD-L1) marker where there was initial response to immunotherapy with a check-point inhibitor but where the tumor was now showing resistance.

3.9 Case 9

We did describe a case of a 66-year-old woman with stage IV non-small cell lung cancer, who not only had the PD-L1 marker, but also her cancer was positive for the epidermal growth factor receptor (EGFR). When her cancer began progressing following chemotherapy with carboplatin, pemetrexed and bevacizumab regimen and the carboplatin and docetaxel regimen, she was started on a targeted therapy for the EGFR marker, erlotinib [45]. At that time, there was only first-generation tyrosine kinase inhibitors.

When her cancer progressed despite erlotinib, she was treated with 11 cycles of the check-point inhibitor nivolumab. It was stopped after 11 months because it was apparent the drug was no longer inhibiting her cancer progression. She qualified for the investigator-initiated study, and thus she was treated with the 300 mg oral daily dose of mifepristone [45].

After 18 months of oral 300 mg single agent mifepristone therapy, there had been no cancer progression based on lung CT scans performed every 2 months. In fact, some lesions were actually smaller. She was considered ECOG 1 at the start of mifepristone therapy. At the end of 1 year, she was still ECOG 1 with a good quality of life and normal physical activity.

After 1 year, her pre-existing severe chronic obstructive pulmonary disease (COPD) worsened and she required supplemental oxygen to keep her po_2 above 80 mmHg. Based on her COPD, but not her cancer which still had not progressed, at

18 months from initiation of treatment, she was an ECOG 3. She died 2 months later from pneumonia.

Thus, this patient not only showed that mifepristone can prolong life and provide a good quality of life not only in a patient whose lung cancer is positive for the PD-L1 marker, but a person who also has the EGFR mutation [45].

Anecdotal cases are important, but more influential to other physicians would be a larger series. Even better would be a controlled trial with sufficient power, and the very best, a study that has all these qualifications, but is also multi-centered. The FDA approved the aforementioned investigator-initiated study for 40 patients. It is not considered ethical to have patients with such severe disease and subject them to placebo controls. Thus, the study was to evaluate in a larger series the efficacy of mifepristone therapy for advanced lung cancer and compare outcome to historical controls, that is, from quality of life to life expectancy, when dealing with a similar group of patients with lung cancer that has stage IV and failed at least two chemo or immunotherapy regimens.

We were allowed two principal investigators. However, as an investigator-initiated study with no funds provided to the principal investigator by a pharmacological company or a grant, we could not find a principal investigator who treats a larger population of patients with lung cancer. Thus, we became, by default, the only principal investigator. Unfortunately, it is not totally clear to us as to the reasons, but despite our efforts we have only recruited the two aforementioned patients that were treated in this investigator-initiated study. Perhaps some of the fault lies in making the criteria for registering too harsh, but most of the problem is that we have not been referred very many patients to even screen for the study. Even the physician who referred us our first case who still is doing so well after almost 5 years of single agent mifepristone therapy, plus years with no side effects, has not referred us another patient [44]. We asked him if he had more patients and he stated that he could send us 40 patients in 1 year, but patients do not want to travel 100 miles every month to receive the medication. This seem unbelievable but this was also related to us by an oncologist whose research with us involving PIBF helped him get into medical school, where the patients would only have to travel only 15 miles. He was supposed to be our first principal investigator, but his associates objected. Even our own well renowned cancer facility at our institution turned down the opportunity to be a principal investigator and has never referred one patient for treating cancer whether they had lung cancer for this investigator-initiated study, or for compassionate use for other cancers. From what we have ascertained, they refer the patients to hospice when they are at the stage eligible for our study. Yet they kindly refer to us many patients to consider oocyte freezing or embryo banking before potential ovary damaging therapy.

3.10 Cases 10 and 11

Actually, there were two patients with lung cancer that we screened that would have qualified for the investigator-initiated study. They both had stage IV non-small cell lung cancer positive for the EGFR mutation that were at the end of targeted therapy (erlotinib, afatinib, and osimertinib) because the lesions were progressing. They both responded very well to single agent mifepristone. Their case reports were accepted for presentation at the 2020 American Association for Cancer Research ("Improvement in quality and length of life following treatment with mifepristone in women with stage IV non-small cell lung cancer positive for the EGFR mutation that previously progressed on targeted therapy"). Because our study was not recruiting very well, we advised these two patients to try compassionate use 200 mg mifepristone, where the drug can be shipped to their

homes, rather than travel thousands of miles monthly to receive the medication gratis as required by the study design.

3.11 Case 12

There were two other abstracts accepted by the annual 2020 AACR meeting. The title of one tells it all – "Treatment with oral mifepristone enables a patient with end-stage pancreatic cancer, in hospice, on a morphine drip, to restore a decent quality of life." The only other patient who we treated with mifepristone from pancreatic cancer, similar to the aforementioned patient, demonstrated a marked relief of her severe pain that had been present despite opiates. However, her husband, a physician, was informed by a major oncologic center of a new phase I research study. He quickly brought his wife there for treatment and she died 2 days later from cardiac complications of the new drug [40].

3.12 Case 13

A third abstract accepted for the 2020 annual AACR meeting is entitled "Palliative benefits of oral mifepristone for metastatic osteosarcoma." This shows the wide diversity of different advanced cancers that have responded to extremely well tolerated oral mifepristone, frequently providing the patients their best quality of life even when their cancers had not been as advanced. The reason is that even in less advanced stages, many of these patients suffered from side effects of chemotherapy or even immunotherapy.

Pancreatic cancer and fibrous osteosarcoma are not known to be associated with the nuclear P receptor. Other patients with some rare advanced cancers have demonstrated significant palliative benefit following mifepristone therapy include a malignant fibrous histiocytoma in a 23-year-old male and an extremely aggressive transitional cell carcinoma of the renal pelvis [40].

4. Clinical studies using mifepristone to treat cancer

4.1 Cancers positive for the classic progesterone nuclear receptor

The presence of the classic nuclear P receptor in breast cancer tumors has been known for at least 40 years [26]. The thinking in those days was that the presence of the hormone receptor may be needed for the tumor to proliferate. Thus, intervening with the hormone receptor interaction may inhibit cancer growth while not creating serious adverse effects in the patient as long as the hormone-receptor interaction was not essential to life or well-being.

Based on the beneficial effects of blocking the estrogen receptor with selective estrogen receptors, that is, tamoxifen, it is not surprising that mifepristone was evaluated for treating advanced breast cancer with the thought that the interaction of progesterone with the classic nuclear progesterone receptor could somehow allow tumors, for example, breast and ovarian cancer to proliferate.

Mifepristone is a type II progesterone receptor antagonist which promotes DNA binding and also promotes progesterone receptor phosphorylation [46]. Mifepristone was given to advanced stage tamoxifen resistant women (second line setting) and the authors reported a complete or partial response in about 10% [47]. However, 6 of the 11 showed stable disease [47]. Another small study found an objective response rate of 18% [48]. For first line, mifepristone for untreated metastatic breast cancer, a 10% objective response rate was observed [49].

The main method of evaluating efficacy of anticancer treatments 25–40 years ago, and even today, is inhibition of disease progression. Thus, the improvement did not seem adequate enough compared to other "more encouraging therapies". Thus, interest waned in treating advanced breast cancer with mifepristone. Subsequently, more experience with mifepristone therapy for a variety of advanced cancers will show that although sometimes the treatment will cause a very good objective remission, the majority of the time the drug provides significant palliation and extension of a higher quality life while it slows disease progression.

For ovarian cancer not only is the classic nuclear progesterone receptor present but it also predicts a favorable outcome [50]. For similar reasoning as with breast cancer, mifepristone was given about 20 years ago to patients with ovarian cancer who had persistent lesions or recurrent lesions despite one round of chemotherapy [51]. Mifepristone 200 mg/day was given daily and continued until disease progression was found. They were treated for a mean of only 2 months. For 34 patients there was a response in 26.5% (9% complete and 17.5% partial) [51]. A second study of this drug conducted 10 years later showed a partial response in 42% of patients [52]. Again, the drug was stopped if there was any evidence of progression. The median time of treatment was 2 months [52]. From what we know today, if they would have continued the drug, the ovarian cancer may have progressed slowly while the patient maintained a high-quality extension of life [53].

5. Discussion

Should biopsy specimens be tested for PIBF to see if a given patient should be treated with mifepristone?

We do not think it would be unreasonable to see if a given specimen produces PIBF, but can we be sure that the tests are sensitive enough to deprive a patient the potential great benefit of treatment with mifepristone?

Can measurement of serum PIBF be helpful in determining if the cancer is responding to mifepristone or if mifepristone therapy is no longer working?

There have been developed more sensitive and specific serum PIBF assays [2]. However, based on measurement of serum PIBF in patients with gynecologic cancers or breast cancers that are P receptor positive, or even associated with breast cancer antigen 1 or 2, the serum level of PIBF may not be helpful for these purposes [54, 55]. It is the PIBF in the tumor microenvironment that seems to be most important, and this, of course, would be difficult to measure.

The 200 mg daily dosage of mifepristone does not appear high enough to block the glucocorticoid receptor. So, another important question, is if it is the action of mifepristone on blocking the P receptor that leads to its efficacy in treating cancer why does it seem to work in cancers that are not associated with the classic nuclear P receptor?

The evidence supports the fact that it acts on membrane P receptors. Activation of the nuclear P receptor initiates transcription, which is a slower process, whereas rapid activation of the membrane P receptor is a more rapid signaling action [46].

Do cancers need to secrete P to activate the membrane P receptor?

It is possible that at a certain stage cancer cells can make P or a P-like substance sufficient to interact with membrane P receptors. There is evidence that a large variety of cancer cells express the human chorionic gonadotropin (hCG)-beta subunit gene [56]. Activation of the hCG beta subunit gene to produce hCG could lead to local P production by the cancer cells. Alternatively, there may be some other mechanism to activate the membrane P receptor to make PIBF. Even with this

scenario, mifepristone could still block the effect of this theoretical non-P membrane P receptor agonist.

Does mifepristone only works when the cancer is at the stage of rapid metastasis?

It is possible that all cancers have mRNA to produce PIBF, but only at a certain level, that is, perhaps stem cell level is the membrane progesterone receptor is activated and PIBF is manufactured. Thus, it is possible that activation of tumor secretion of PIBF only occurs at the stage when it is ready to rapidly metastasize. About 20% of meningiomas are associated with the classic nuclear P receptor. However, a large study comparing mifepristone vs. placebo for unresectable tumors did not find any therapeutic benefit for mifepristone vs. placebo [57]. This could be because meningiomas are slow growing tumors and the PIBF mechanism is only seen with rapidly growing tumors. However, it is also possible that some meningiomas are considered benign. Thus, maybe it is the ability to make PIBF that is one factor allowing the tumor to follow a benign vs. malignant course. One benefit of this large study was to demonstrate a very good safely profile for mifepristone with few side effects [57].

Since a compassionate use IND is required by the FDA, that organization is reluctant to grant an off-label use unless all "standard" treatments have been exhausted. Thus, most of the study subjects in our center have been patients with very advanced cancers where there are few, if any, reasonable treatment options.

One exception is a man, who at the age of 58 was found to have bilateral renal cell carcinoma with metastases to local lymph nodes [42]. Renal cell carcinoma can be multifocal, and even when several lesions are present, the tumor is generally not extremely aggressive. Today the recommendation is renal sparing surgery and to remove the tumors every time one reaches a certain critical size [58–60]. But 16 years ago, the recommendation was bilateral nephrectomy.

Since there were no chemotherapy or immunotherapy agents16 years ago for renal cell carcinoma, and the patient did not want to become a dialysis cripple, the FDA approved a compassionate use IND for oral mifepristone following a laparoscopic hemi-nephrectomy with retention of a kidney with three lesions left untreated.

After 10 years of single agent treatment, there were no new tumors. The three lesions previously noted on the left kidney remained stable [42]. After 10 years his diabetes caused kidney failure and the start of dialysis. Thus, he had the 1½ kidneys removed. After 2 years of hemo-dialysis, he was approved for a kidney transplant. He is still doing well 16 years from initial diagnosis [42].

This case showed that mifepristone can also work to inhibit tumor growth even when not at the rapidly growing cell stage. Whether this is specific only for renal cell carcinoma, or applies to other malignancies, needs to be determined. Thus, perhaps one should consider using mifepristone in earlier stage metastatic cancers following tumor remission following treatment with chemotherapy or immunotherapy to possibly inhibit recurrence or negate the need to treat with another chemotherapy or immunotherapy regimen with morbid side effects.

One final thought. Frequently, once a tumor has widely metastasized chemotherapy or even immunotherapy may frequently extend life somewhat at the expense of significant side effects from treatment. Mifepristone therapy is devoid of major side effects, and thus may provide possibly a longer higher quality life than "approved therapy." The treatment of patients with cancer has provided huge profits both for the pharmaceutical companies and the treating institutions. So realistically it is unlikely that mifepristone therapy will become popular in capitalistic societies.

However, in some countries needed to provide effective, yet inexpensive treatment, one could consider offering patients oral government provided mifepristone rather than expensive chemo or immunotherapy agents. The cost of a mifepristone pill in China is 50 cents. In fact, since growth of tumors is still consistent with a prolonged good quality life, one could save money on expensive diagnostic tests to monitor progression. Possibly mifepristone could be considered first line therapy for metastatic disease with consideration of other therapeutic modalities only if health deteriorates despite mifepristone therapy.

Since the drug is available as a generic already, it is unlikely any pharmaceutical company will invest in larger studies to prove its efficacy. Hopefully, the published anecdotal cases, and the easing of the requirements for compassionate use, will encourage other clinicians treating patients with advanced cancer to try the drug and publish their findings. If enough treating physicians request compassionate use IND for mifepristone use, perhaps the FDA will eventually drop the requirement of compassionate use IND, facilitating the use for treating physicians around the world. Many countries, similar to the United States, at this time also restrict the use of mifepristone solely for the purpose of therapeutic abortions, and in some countries, it is completely illegal, at least at the relatively inexpensive price for the 200 mg dosage to use this drug. The use of the 300 mg dosage that does not require a compassionate use IND is cost prohibitive. Possibly the manufactures may one day reduce the price considerably or it will be manufactured by a generic company at a much lower price when the patent expires. Perhaps at a lower cost, insurance companies will be happy to pay for off-label use of mifepristone realizing how much cheaper it is for cancer therapy than conventional chemo or immunotherapy regimens.

As previously mentioned, clinical trials with mifepristone for cancers associated with the classic nuclear P receptor were "disappointing" and thus clinical trails were not pursued. When these studies were initiated 20–30 years ago, the hope was that metastatic cancer can be "cured." It is now realized that the best hope for advanced cancer is a truce with extension of a better quality of life. Also, at that time the goal of therapy was to induce a tumor response as evidenced by complete or partial tumor regression. We think if they had used the endpoints of quality and length of life, they would have had the satisfaction of treatment as we have had in these anecdotal cases. The majority of cases do not show tumor regression but stable disease and improved quality and length of life.

As far as side effects, the drug has been well tolerated. In higher dosages mifepristone can, by blocking the glucocorticoid receptor, lead to higher serum cortisol levels which acts on the mineralocorticoid receptor leading to hypokalemia. One has to be careful when using other drugs that can interfere with the metabolism of mifepristone leading to hypokalemia. We had one unreported case of a woman adding mifepristone to her ongoing treatment with alpelisib, which in itself can cause hypokalemia. Whereas the combination led to hypokalemia, neither drug by itself caused it. She was taking just the 200 mg dosage of mifepristone.

Similarly, case number 9, who was taking the 300 mg dosage, did develop hypokalemia when she was switched to another bronchodilator for her COPD, but reverted back to normal when it was stopped. She was taking the 300 mg dosage of mifepristone [45].

One man with stage IV non-small cell lung cancer became more somnolent when adding mifepristone to his fentanyl that he was using for pain. Though we advised him to reduce the dosage of fentanyl, he chose to just stop the mifepristone and died 2 weeks later. He had only taken the mifepristone for 2 days.

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Integrating Immunotherapy with Chemotherapy: A New Approach to Drug Repurposing

Hina Qayoom, Umar Mehraj, Shariqa Aisha, Shazia Sofi and Manzoor Ahmad Mir

Abstract

Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype lacking the three hormonal receptors namely estrogen receptor, progesterone receptor and HER2 receptor, and the only treatment option available for TNBC is chemotherapy. Chemotherapy lacks specificity since it acts on normal healthy cells as well resulting into secondary diseases in TNBC patients. In addition chemotherapy poses recurrence and relapse issues due to the development of chemoresistance among TNBC patients. Immunotherapy remarkably immune checkpoint inhibitors show a great therapeutic potential in TNBC. As TNBC contain an increased TILs (tumor infiltrating lymphocytes) infiltration making it more suitable as a therapeutic target anti-tumor immune strategy. Moreover, evidences have indicated that chemotherapy upregulates the anti-tumor immune response in TNBC. As a result, a combination of immunotherapy with chemotherapy may increase the overall relapse and recurrence free survival of TNBC patients. Therefore, in this Chapter we will focus on how the immunotherapy works in TNBC, their effects and consequences. We will further be discussing the clinical studies and the importance of immune checkpoint inhibitors (ICIs) in combination with various therapeutic agents and target. Further, we will explore the processes involved.

Keywords: TNBC, PD-1, immunotherapy, immune checkpoints, immune checkpoint inhibitors, epigenetics, CTLA-4, oncolytic virus

1. Introduction

Triple negative breast Cancer (TNBC), is an aggressive breast cancer subtype characterized by the lack of hormone receptors; estrogen receptor, progesterone receptor and HER2 receptor accounting for about 15–20% of all breast cancers, with chemotherapy available as the prime systemic therapy. The treatment results into low median overall survival with earlier recurrence and metastasis posing to be a great hurdle in the control of this disease [1]. Therefore, improved therapies are urgently needed. Immunotherapy has prolonged survival in other solid tumors and represents a promising treatment strategy for TNBC (**Figure 1**). In the recent days, targeting immune checkpoint inhibitors are noted immunotherapeutic agents that are known to block immunosuppressive receptors like PD-1 (anti-programmed

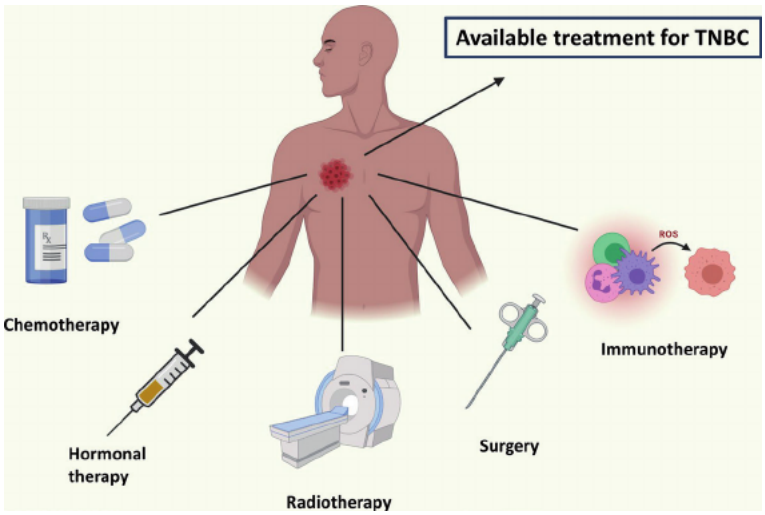

Figure 1.
Represents the available treatments for TNBC (triple negative breast cancer).

death receptor-1) and CTLA-4 (cytotoxic T lymphocyte antigen-4), which are significantly involved in tumor directed immune responses [2]. Moreover, several characteristics of TNBC make immunotherapy to be corner stone of the modern therapeutic regimens such as the presence of TILs (Tumor infiltrating Lymphocytes (TILs). The TILs are associated with better therapeutic responses increasing the disease free survival and overall prognosis in TNBC in comparison to other breast cancer subtypes. The presence of TILs as well acts as predictive biomarkers for immunotherapy response that makes immunotherapy more intriguing for TNBC treatment [3–5]. Besides, TNBC are known to possess higher PD-L1 expression levels on both tumoral and immune cells that are likely to respond to the immune checkpoint inhibitors (ICIs) such as pembrolizumab, nivolumab (monoclonal antibodies against PD-1), Ipilimumab (antibody against CTLA-4) and Atezolizumab, Avelumab (antibody against PD-L1) [2, 6, 7]. In addition, the presence of significant number of non-synonymous mutations in TNBC generate neo-antigens specific to tumors that activate robust anti-tumor immune responses that can be synergistically utilized by the current immunotherapeutic agents like ICIs [8–10]. Nevertheless, the presence of higher levels of BRCA1 and BRCA2 mutations giving rise to unstable genetics acts as a significant predictive marker for immunotherapy response [11].

The immune system plays a dual role in a way that it not only is involved in tumor initiation and progression but also acts significantly in the recognition and destruction of cancer cells. The later generates a tumor-directed immune response involving cytotoxic T lymphocytes [12, 13]. For cancer progression the tumors are known to evade the anti-tumor immune response by certain array of mechanisms like activation of pro-tumor-polarized innate inflammatory cells, activation of humoral immunity, suppression of tumor-specific antigens, infiltration by Th2 T cells, absence of major histocompatibililty complexes (MHC) on tumor cell surface and negative immune checkpoint inhibitor expression by tumor cells [13, 14]. These mechanisms followed by tumor cells to evade immune responses are known as hallmarks of cancers as these work in concordance to suppress the anti-tumor response and promote cancer progression. Therefore, in order to bring cancer control strategies targeting these specific mechanisms are utilized in immunotherapy to bring in control the tumor progression (**Figure 2**) [15].

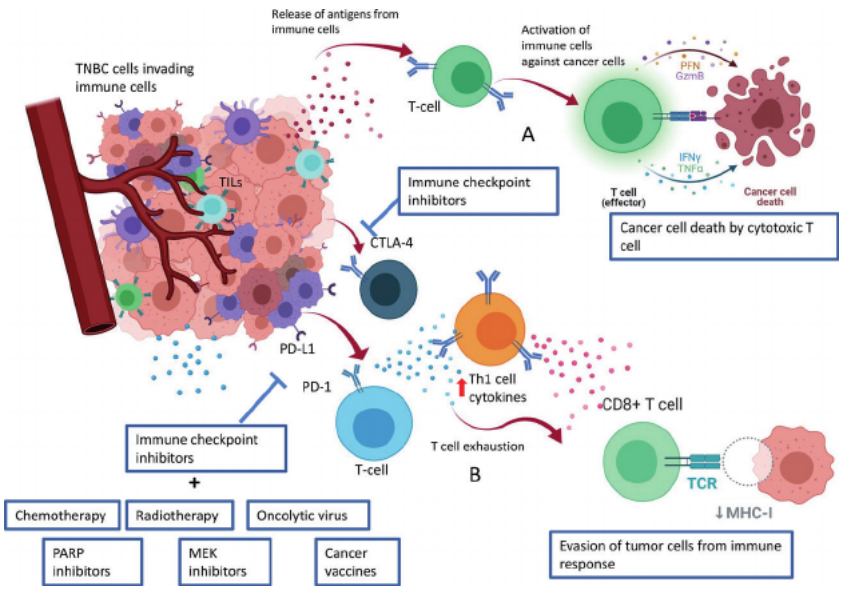

Figure 2.
Overview of involvement of immune system in TNBC with combination treatment options; A. Represents on recognition of the antigen from the tumor cell the immune cell destroying the tumor cell B. Shows that how PD-L1 from the tumor cell interacts with PD-1 and this binding causes T cell exhaustion and helps the tumor cell evading the immune response.

Therefore, actively manipulating the immune system for TNBC treatment represents to be an attractive strategy as this particular breast cancer subtype has lacked in terms of extensive clinical management. In view of that, immune checkpoint inhibitors (ICIs) has revealed promising results in TNBC patients by substantial improvement in TNBC patients overall prognosis. However, the focus of this field is to recognize the immunogenic identity of patients for the clinical management of patients and in specific to identify specific therapeutic agents to target tumor microenvironment [14]. Nevertheless, the utilization of current therapeutics like chemotherapy, radiotherapy in combination with immunotherapy will augment the immunotherapeutic response as they enhance tumors mutational load, downregulate immune suppression by tumor microenvironment and boost antigen presentation by tumor cells, henceforth making tumors more prone to immunotherapy (**Figure 2**) [16–18]. Interestingly, many clinical trials are underway and some have revealed that combination of immunotherapy with other therapeutic agents besides chemotherapy and radiotherapy has enhanced the patient responses in terms of progression free survival and standard of care [19, 20].

2. Role of immunotherapy in TNBC

The immune system is known to kill tumor cells by a process called immunosurveillance in which the immune cells target and kill the tumor cells by two ways; either directly or indirectly by releasing soluble chemicals. The cells involved are cytotoxic T lymphocytes (CTL), dendritic cells (DC), macrophages, Natural killer cells (NK) etc. As described earlier, the cancer cells are known to evade the host's immune responses in that the host's immune system identify the tumor cells as self due to which the tumor cell is favored to escape, grow, proliferate and metastasize to distant organs. Furthermore, as the tumor develops, they modify the immune cells for their own benefit like they modify TAMs and recruit them to the tumor

microenvironment to release chemicals that suppress the immune system further enhancing the suitable environment for the tumor cells to survive and proliferate [21]. Therefore, targeting this strategy of immune evasion by cancer cells i.e. modulating the immune system is imperative for the development of therapeutics against tumors. In addition, the currently available treatment options like chemotherapy, radiotherapy are known to be ineffective because of the induction of relapse and recurrence, development of resistance, lack of specificity in addition to side effects and toxicity that leads to tumor development and metastasis in secondary sites. In view of this, immunotherapy is considered to be the most reliable therapeutic approach in terms of target specificity by targeting different immune cells, their functional attributes to block the development and spread of aggressive tumors and as a non-toxic anti-cancer therapeutic strategy. Moreover, immunotherapy has emerged as the fourth most important treatment for cancer after surgery, chemotherapy and radiotherapy and has shown effective treatment responses among patients (**Figure 2**) [22].

Recently immunotherapy was developed as an effective treatment strategy against cancers with a goal to design therapeutics that can effectively enhance the immune system in terms of its specificity and strength its response towards the evading tumors [23]. In the year 2018, James P. Allison and Tasuku Honjo won the Nobel Prize in Physiology and Medicine for discovering a treatment for cancer by downregulating the negative immunomodulation. In their study, they demonstrated that the immune checkpoints like PD-1 (programmed cell death protein1) and CTLA-4 (cytotoxic T lymphocytes associated protein 4) act as "brake" in immune system as they may reactivate T cells by immune checkpoint inhibition, hence eliciting an improved immune response against malignant tumors [24]. The significance of immune checkpoint inhibitors as potential therapeutics has proven in various studies. Many studies have revealed that PD-1 inhibition promotes effective immune responses against cancers [25]. Accumulating studies on PD-1 signaling suppression has revealed that the patient's clinical response to immunotherapy depends upon the effectiveness of T-cells to penetrate the tumor [26]. In the past decade many immune system components have been explored as adoptive immunotherapies like cytotoxic T cells, TILs, anti-CD3 monoclonal antibody-induced killer cells and activated killer cells but they showed less efficiency as therapeutics because of their low anti-tumor functions [27]. However, an *in-vitro* study has suggested the cytokine-induced killer (CIK) cells to a promising target for utilization as immunotherapeutic target because of its higher proliferation rate, hence more effectiveness towards eradicating cancer [21]. CIKs contribute to sturdy cytolytic activities towards tumors as these are non-major Histocompatibility complex- restricted cells that can express both natural killer cell and T cell markers such as CD56 and CD3 [28]. Furthermore, CIKs are known to improve the immune response in patients by regulating and therefore, increase the efficacy of immune function [29]. However, study of CIK cell therapy in breast cancer, particularly in TNBC has been limited. Despite that evidences have reported that the association of CIKs with chemotherapy may result in synergistic effects, supported by an *in-vitro* and *in-vivo* study against cancer stem cells that were resistant to chemotherapy. Therefore, strongly suggests that combined therapy might improve therapeutic efficacy in patients having TNBC, as chemotherapy has shown to regulate the patient's immune status [30].

3. Immune checkpoints in immunotherapy

Immune checkpoints comprise of a collection of different regulatory proteins in the adaptive system that regulate the immune system functions i.e. anti-tumor

activity and self-tolerance. They are known to function by coordinating the frequency, magnitude and type of immune response either via positive or negative regulation. There are mainly two immune checkpoint s studied namely PD-1/PD-L1 and CTLA-4, as their presence in the TME prevents to elicit an anti-tumor response via negative regulators of immune activation [31].

3.1 PD-1

PD-1 also known as CD279 was first discovered in the year 1992 [32]. It is a 55 kDa transmembrane protein comprising of 288 amino acids with an extracellular N-terminal domain, a cytoplasmic tail at each N and C end, a transmembrane domain respectively with two tyrosine bases [33]. PD-1 are expressed on a number of immune cells like macrophages, B lymphocytes, activated T cells, Dendritic cells, natural killer cells, activated T cells and monocytes. However, they are highly expressed on specific T-cells. PD-1 is known to act as an inhibitor of both innate and adaptive immune responses [34]. It is supposed its transcription is triggered by many transcription factors such as NOTCH, nuclear factor of activated T cells (NFAT), Interferon (IFN), Forkhead box protein (FOXO1) and interferon regulatory factor 9 (IRF9) [35]. PD-1 expression is highly increased during acute infection and also when there happens to be leakage from cancer cells. PD-1 function in both beneficial and harmful manner to the immune system as it plays a significant role in maintaining immune tolerance by regulation of the harmful and inefficient immune responses while also interfering with the classical protective role of immune system by negative regulation [36–38]. A higher PD-1 expression has been seen in TNBC patients in comparison to non-TNBCs and has been associated with larger tumors, higher histological grades, increased TILs etc. [39].

3.2 PD-L1

PD-L1 is a ligand to PD-1. It belongs to the B7 series and is also known as B7-H1 and CD279. It is a transmembrane glycoprotein as is PD-1, containing

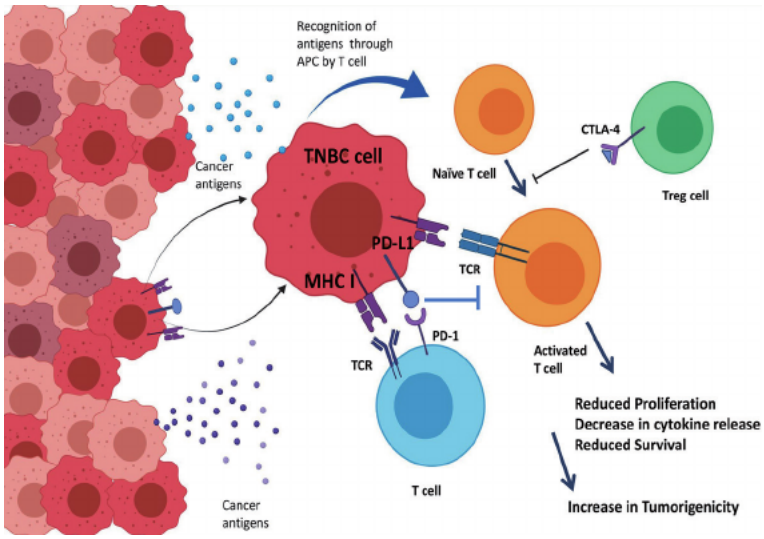

Figure 3.
Represents PD-1 mediated T cell inhibition. The binding of PD-L1 expressed on tumor cells binds to its receptor PD-1 on T cells delivering an inhibitory signal to T cells that lead to T cell exhaustion and ineffective T cells.

Similarities	Difference
Expressed by activated T cells	CTLA-4 limits T-cell responses early in an immune response, primarily in lymphoid tissues; PD-1 limits T-cell responses later in an immune response, primarily in peripheral tissues
Regulate an overlapping set of intracellular T-cell signaling proteins	CTLA-4 affects Tregs functioning; the role of PD-1 on Tregs is unclear
Level of expression affected by the strength and duration of TCR signaling	CTLA-4 is expressed by T-cells; PD-1 is expressed by T cells and other immune cells
B7 receptor family members	CTLA-4 ligands are expressed by professional APCs; PD-1 ligands are expressed by APCs and other immune cells, and can be inducibly expressed on non-immune cells, including tumor cells
Reduce T-cell proliferation, glucose metabolism, cytokine production and survival	PD-1 engagement interferes with more T-cell signaling pathways than does CTLA-4 engagement

Adapted from [40].

Table 1.
Comparison of immune checkpoints CTLA-4 and PD-1.

290 amino acids with IgC domains in its extracellular portion. The cells that express PD-L1 include: activated B and T cells, epithelial cells, macrophages and dendritic cells, particularly at the time of inflammatory responses. The PD-L1 expression is connected with the production of Th1 cytokines, presence of CD8 T cells, interferon, other chemical factors as well as expression of specific genes i.e. all these are responsible for the over expression of PD-L1 and further malignant disease progression, which we will be discussing later in the chapter. Therefore, inhibiting the particular pathways for instance, on activation the NK and T cells secrete interferon-gamma that induces PD-L1 expression on the cells including tumor cells etc. has been shown to promote strong antitumor responses among patients.

The PD-L1 is utilized by the opportunistic tumor cells to evade immune response by mimicking the "Adaptive immune process". Furthermore, PD-L1 is known to act as a pro-tumorigenic factor activating survival and proliferating signaling pathways by receptor binding, hence implicating its greater role in tumor proliferation and metastasis (**Figure 3**). In addition, PD-L1 also acts in a non- immune pattern by inducing epithelial to mesenchymal transition exerting in the tumor cells stem cell like characteristics promoting metastasis and disease progression **Table 1** [41].

3.3 CTLA-4

CTLA-4 is a member of the CD28 family and is considered to be the "leader" of the immune checkpoint inhibitors as it potentially stops autoreactive T cells in the lymph nodes at the initial stages of development [42, 43]. It is the first immune checkpoint discovered among other immune checkpoints. It is a transmembrane receptor of T cells and it is a leukocyte differentiation antigen that regulates the immune process by negative regulation by competing and binding to the B7 receptor, as it is a CD28 homolog [40]. CTLA-4 plays a significant role to prevent self-reactive immune responses particularly by increasing immunosuppressive Treg. Activity and downregulation of the T effector cell function [14].

4. Possible mechanism of action of anti-programme death receptor-1/ Ligand-1 (PD-1/PD-L1) in cancer

PD-1/PD-L1 is known to control the induction and maintenance of immune tolerance within the tumor microenvironment. It performs a significant role in cytotoxic secretion and T cell activation and proliferation in cancer to inhibit anti-tumor immune responses in host [41]. During tumor proliferation, the PD-L1 is highly expressed on tumor cells that bind to the PD-1 receptor on T cells that receive an inhibitory signal from the PD-L1 binding i.e. to inhibit the T cell's immune function that leads to T cell exhaustion making T cells ineffective (**Figure 3**).

However, monoclonal antibodies that target PD-1 and PD-L1 are being studied and used as these pathways are majorly taken by tumor cells to proliferate in host's body that are known to typically regulate activity of T cells for their own benefit that is to evade the immune responses generated against them. Accumulating evidences has suggested that by inhibiting the binding of PD-L1 to PD-1, the anti-tumor response is made stronger as the T cell exhaustion is reversed. Therefore, in view of that several monoclonal antibodies are being studied, particularly in TNBC like Atezolizumab, Avelumab and Durvalumab that specifically target PD-L1 and others such as Pembrolizumab and Nivolumab specific to target PD-1 [31].

5. Possible mechanism of action of cytotoxic T lymphocytes (CTLA-4) in cancer

CTLA-4 (Cytotoxic T lymphocyte-associated protein-4), is another regulatory pathway of T cells. During T cell activation CTLA-4 is highly upregulated. Upon T cell activation, the CTLA-4 is translocated from the intracellular granules to the plasma membrane that further amplifies the T-cell response by regulating T-cell priming and activation. It inhibits the intracellular T cell activation signaling by competitive binding for CD80/CD86 that results in downregulation of immune response. Moreover, it acts through protein tyrosine phosphates 6 and 11 to suppress

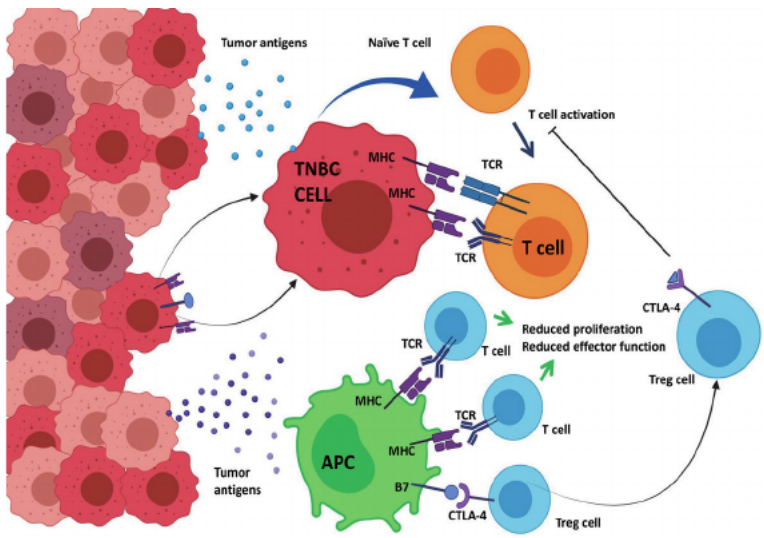

Figure 4.
Shows CTLA-4 inhibits T cell activation thereby regulating the immune responses. Therefore, tumor cells escape immune response by suppressing CTLA-4.

the TCR signal. CTLA-4 plays an important role in regulating peripheral tolerance that is an immunological process to prevent auto-immune responses by suppressing T effector cell function and further by upregulating the immunosuppressive Treg activity. Tregs express CTLA-4 constitutively unlike effector cells thereby acting as a major mechanism for immune suppression (**Figure 4**) [14].

Therefore, many monoclonal antibodies are currently being studied for instance; Tremelimumab specific for target CTLA-4 is being investigated in patients with TNBC. Limited research is available regarding CTLA-4, eagerly awaiting the need for research in discovering treatment options and other potential targets in TNBC treatment [31]. By inhibiting the CTLA-4 mediated response or the blockade of CTLA-4 results into the activation of non-specific immune cell activation and is connected with increased treatment-related adverse events (TRAEs). For instance, CTLA-4 depletion has results into rheumatoid arthritis, type I diabetes, collagen induced arthritis and systemic lupus erythematosus [14].

6. Other immune checkpoints under investigation

Apart from the above two immune checkpoints, a variety of other immune stimulatory and suppressive checkpoints are currently under investigation as immunotherapy targets that include; TIM-3, LAG-3, TIGIT, VISTA and BTLA-4, these reduce the anti-tumor immune response by regulating T cell activity like CTLA-4 and PD-1. Among them TIM 3, BTLA-4 and LAG-3 are implicated as T cell exhaustion markers in tumors same as that of PD-1. TIM 3 negatively regulates the cytotoxic CD8 T cells and Th1 CD4 T cells, thereby shifting the immune responses. TIGIT is expressed by a number of cells such as: T cells, Treg cells and NK cells

Immune checkpoints	Function
Immunoinhibitory checkpoints	
PD-1	Regulates T cell activation by binding to its ligand PD-L1 and PD-L2
CTLA-4	Acts by competitive binding with the receptors and prevent the co-stimulatory signal thereby balancing the stimulatory signals of the host immune response
TIM-3	Shifts the immune response by negatively regulation of Th1 CD4 T cells and cytotoxic CD8 T cells
TIGIT	TIGIT on T cells binds with the poliovirus receptor on the APCs and act as competitive antagonist to CD226 have suppressive effects
VISTA	Expressed by both APCs and T cells plays a role in both Treg function and myeloid cell activation
Immunostimulatory checkpoints	
ICOS	It is a member of the CD28 family. It provides the second signal in immune activation by binding with B7H/B7RP-1.
CD40L	CD40L interacts with CD40 receptor on T cells and function by promoting a proinflammatory immune response
OX40	OX40 downregulates Treg function by binding with the ligand OX40L. It also induces the expression of pro-apoptotic proteins like BCL-2 and BCL-XL

Table 2.
Immune checkpoints in immunotherapy.

and is known to bind to poliovirus receptor on APCs or tumor cells. It is supposed to perform both direct and indirect immunosuppressive effects by competitively binding to NK and T cell receptors in place of CD226; it also leads to downstream inhibition of AKT signaling in T cells [14, 44]. In addition, TIGIT increases the suppressive activity and releases inhibitory cytokines by receptor binding of TIGIT on the APCs and Tregs [45]. Moreover, VISTA is expressed by both APCs and T cells play a role in both Treg function and myeloid cell activation [14].

On the contrary, other checkpoints like OX40, ICOS and CD40L are immunostimulatory checkpoints that function in the maintenance and activation of effector T cells. The expression of OX40 is induced at the time of T-cell activation leading to the expression of anti-apoptotic factors such as BCL-2 and BCL-XL that leads to the sustenance of T cells proliferation. It also acts as a co-stimulatory signal in tumor induction and is constitutively expressed on Tregs and OX40 also decreases the Treg function by binding to its receptor on Treg [46–48]. Furthermore, ICOS leads to the activation of second signal in immune activation by binding to B7H/B7RP-1 [49]. Another immunostimulatory checkpoint CD40L interacts with CD40 on APCs induces via NF-ƙB signaling a proinflammatory immune response **Table 2** [14].

The immunostimulatory checkpoints can be inhibited by therapeutic agents targeting recombinant ligand peptides, ligands expressing viral particles or agonistic monoclonal antibody that is in contrast, to the inhibitory checkpoints where monoclonal antibodies inhibit the interaction between the respective ligand and receptors. Therefore, there is an emerging need to fully explore these biomarkers for better prognosis of patients using immunotherapy strategy [14].

7. Biomarkers in immunotherapy

Biomarkers are of significant importance in view that it predicts the clinical benefit to immunotherapy. Therefore, there is a need to bring into light several biomarkers in TNBC to distinguish that which patients is likely to get benefited from the ICIs or to build up certain therapies to overcome the hindrance in treating the respective malignancy. Until now PD-L1 was considered to be the major biomarker in TNBC. However, recent studies depicted that most of the mTNBC patients are PD-L1 negative arising the need to prospect into the immunotherapy field to find other novel biomarkers to get an insight into the patient responses to immunotherapy as a monotherapy or as a combinational therapy [50, 51]. Some of the biomarkers studied so far in TNBC include: TILs, TMB (tumor mutational burden), Gene signatures.

8. Tumor infiltrating lymphocytes as biomarkers (TILs)

Tumor infiltrating lymphocytes (TILs) have a predominant role in breast cancer as predictive and prognostic biomarker. It is present intratumorally and in adjacent stromal tissues. The increased presence of TILs in Breast cancer is associated with improved prognosis and overall survival in response to neoadjuvant chemotherapy [52, 53]. In a recent study higher number of TILs was in TNBC as compared to other breast cancer subtypes, therefore is associated with the possibility to show better responses to neoadjuvant and adjuvant chemotherapy with relapse free survival [54, 55]. The connection of TILs with anti-tumor immune response in TNBC patients also serves as a predictive biomarker, thereby making examination of immunotherapy in TNBC more interesting [14]. Furthermore, clinical trial KEYNOTE-173 trial investigating pembrolizumab in combination with chemotherapy has shown

promising results in the neoadjuvant setting of TNBC, as this trial demonstrated the presence of higher levels of TILs and higher PD-L1 expression resulting in a high combination score with increased overall response rates in TNBC patients [56].

9. Tumor mutational burden (TMB) as TNBC biomarker

TMB is defined as the measurement of non-synonymous mutations present in tumor cells. Here mutations lead to enhanced expression of neoantigens in terms of MHC I class antigens thereby increasing the visibility of cancer cells to T cells. However, limited data for TMB is reported while the frequencies of TMBs are found to be significantly higher in TNBC comparative to the other breast cancer sub types [57]. Therefore, the presence of TMB is linked with immunogenicity in several tumor types [58]. A recent study revealed no significant difference for breast cancer patients pre-treated with ICIs (immune checkpoint inhibitors) in survival. Therefore, it is assumed that TMBs alone are not supposed to represent a sole predictor as biomarker evoking the need to enrich the available information regarding TNBC biomarkers [58, 59].

10. Gene signatures as biomarkers in TNBC

A number of multiple gene signatures in correlation with TILs have been studied as surrogates of breast cancer immunogenicity. According to immune-related gene expression profiling breast cancer consisted of four categories namely ICR1–4 (immunologic constants of rejection) and these were seen to be associated with survival in a retrospective manner using in-silico analysis. Interestingly, the ICR4 (Th1 helper phenotype) was linked with the upregulation of transcripts like PD-L1, IDO1, PD-1, FOXP3 and CTLA-4 that indicated a better survival among patients, in contrast a negative regulation was showed in disruptions induced by the presence of MAPK components linked with the ICR1, an unfavorable-immune response. A study on mouse models has shown an increased anti-tumor immune response in TNBC patients that was suggested to result by the inhibition of MEK, a molecule of MAPK pathway in combination with PD-1 inhibitor due to which the MHC I and PD-1 expression on Tumor cells increased resulting in apoptosis of cancer cells. Moreover, in TNBC a four gene-signature such as CXCL13, GBP1, SULT1E1 and HLF were shown to represent an upregulation of TILs and enhanced disease free survival among patients, however their predicting response with ICIs needs to be defined [58].

11. Importance of immune checkpoint inhibitors as monotherapy

Immunotherapy stimulates the immune system by active immunization with cancer vaccines or passive immunization with tumor-specific antibodies and immune modulators, such as immune-checkpoint inhibitors. Immune checkpoints are a complex group of adaptive immune system regulatory points that play roles in self-tolerance and antitumor immunity. These checkpoints regulate the immune response in either a negative or positive way, coordinating the magnitude and form of response. Immune checkpoint inhibitors (ICIs) are regarded as the emerging immunotherapy superheroes, allowing a patient's self-immune cells to destroy tumors and remodeling cancer treatment in a board spectrum of cancers. The use of immune checkpoint inhibitors against programmed death receptor-1 (PD-1) or its ligand PD-L1 to treat a wide range of solid and hematologic tumors has dramatically altered the cancer treatment paradigm.

11.1 PD-1 inhibitors

PD-1, also known as CD279, is a CD28 family member expressed on lymphoid cells such as T cells, B cells, and natural killer (NK) cells, as well as on myeloid cells [60]. The binding of PD-1 on T cells with the ligands PD-L1 or PD-L2 suppresses signals downstream of T-cell receptor activation in the context of antitumor immunity [61, 62]. The monoclonal antibody that target the programmed death-1 receptor is Pembrolizumab, which is a humanized monoclonal antibody directed against PD-1.

11.2 Pembrolizumab

Pembrolizumab prevents immune-cell deactivation and inhibition by sterically blocking the interaction of PD-1 and its ligands. Pembrolizumab was the first immune checkpoint inhibitor to be approved as a first-line treatment, as well as the first PD-1-targeted therapy. Pembrolizumab a dose of 10 mg/kg was administered every two weeks to patients with previously treated, advanced TNBC in the KEYNOTE-012 trial, which showed efficacy and an adequate safety profile [63]. The overall response rate was 18.5 percent of the 27 patients who were assessed for antitumor activity, with 17.9 weeks an average response time (**Table 3**) [63]. The KEYNOTE-086 trial is presently examining the use of pembrolizumab (200 mg per 3 weeks) in metastatic TNBC (NCT02447003). Cohorts A and B were presented in an oral session at the 2017 ASCO conference [64, 65]. Cohort A comprises of patients with TNBC who had advanced on at least one systemic treatment. Among the 170-patient cohort, 4.7% responded, and 7.6% accomplished disease control for 24 weeks or longer, which included stable disease, partial response, and complete response [66]. In addition, 0.6% showed an absolute response to pembrolizumab monotherapy, and 27% had a decrease in the target lesion size after the first dose. The KEYNOTE-086 trial's Cohort B included metastatic TNBC with PD-L1+ tumors, without having received some systematic treatment previously. 23% of the 52 patients in this cohort showed an objective responses [64]. The use of pembrolizumab as a primary therapy and the inclusion of PD-L1+ tumors as a criterion for inclusion may have contributed to the increased response in cohort B, with only 58 percent of the patients admitted had a cumulative PD-L1 positive composite score of >1 (**Table 3**) [64].

11.3 PD-L1 inhibitors

The monoclonal antibodies atezolizumab and avelumab target the PD-L1, a transmembrane protein found on tumor cells. Avelumab is a fully human IgG1 MAB that binds to PD-L1, while as Atezolizumab is a humanized IgG1 isotype

Agent	Clinical trial id	Cancer type	Phase	Recruitment status
Pembrolizumab	NCT01848834	mTNBC	Ib	Completed
Pembrolizumab	NCT02447003	mTNBC	II	Completed
Atezolizumab	NCT01375842	mTNBC	I	Completed
Avelumab	NCT01772004	mTNBC	Ib	Completed
Tremelimumab	NCT02527434	mTNBC	II	Active, not recruiting

Table 3.
Main monotherapy clinical trials of immune checkpoint inhibitors in mTNBC.

monoclonal antibody that binds to PD-L1. The FDA has approved these PD-L1 inhibitors for the treatment of other solid tumors, and they are currently being explored further for the treatment of TNBC.

11.4 Atezolizumab

The first PD-L1 inhibitor to receive FDA approval was atezolizumab. An open-label, phase I dose-escalation analysis (NCT01375842) showed that Atezolizumab is safe in patients with locally advanced or metastatic solid tumors (**Table 3**). A cohort of 54 patients with mTNBC was evaluated for protection, and 21 patients were evaluated for efficacy in this study. 69% of the patients in the protection cohort had PD-L1 expression of at least ≥5%, and all of the patients in the efficacy cohort had PD-L1 expression of at least ≥5%. The ORR for this study was 19%. There were three patients who had pseudoprogression, but their tumors gradually shrink. A total of 63% of patients experienced drug-related side effects, with 11% experiencing grade 3 toxicity. Pneumonitis of grade 4 was diagnosed in one of the patients. Fatigue (15%), fever (15%), and nausea (15%) were the most common drug-related side effects [67].

11.5 Avelumab

In a Phase 1b JAVELIN trial, a human anti-PD-L1 IgG1 mab, Avelumab, was tested in patients with MBC [68] (**Table 3**). A total of 168 MBC previously treated patients were treated with avelumab monotherapy, including 58 TNBC patients. The confirmed ORR for the whole population was 3%, with 1 CR (complete response) and 4 PRs (partial responses). The ORR for TNBC patients was 5.2 percent. Furthermore, in both general population (16.7% vs. 1.6%) and in TNBC class (22.2% vs. 2.6%) patients with PD-L1 positive tumor-associated immune cells had a greater ORR than those with PD-L1 negative tumor-associated immune cells.

11.6 CTLA-4 inhibitors

CTLA-4 inhibits T-cell activation by interacting with its target ligand, CD80 or CD86 [69, 70]. Monoclonal antibodies (mAbs) that block CTLA-4 have been demonstrated to augment T-cell activation and thereby enhance cancer cell death.

11.7 Tremelimumab

A phase II open-label trial (NCT02527434) is evaluating the efficacy of Tremelimumab, a CTLA-4 inhibitor, in patients with advanced solid tumors such as TNBC (**Table 3**). While on treatment with tremelimumab, if the patient's develops progression in disease, they are given Durvalumab or a Durvalumab/Tremelimumab in combination. The objective response rate is the primary endpoint, with length of response, progression-free survival, and overall survival as secondary endpoints [71].

12. Drug repurposing an important aspect in immunotherapy regimen

Despite the success of disease diagnosis in modern era, the recent developments and discovery of new drug is laborious, inefficient, time consuming process and costly process [72, 73]. Not only that most drugs face high failure rates in clinical trials [74]. To overcome these problems in drug discovery a strategy namely drug repurposing (also called drug reprofiling or repurposing) came into existence which works by identifying existing drugs and using them for new purposes [75]. Several strategies

are being put to use in order to repurpose the existing drugs whether FDA approved or which are used under investigation. These include methods based on computational and non-computational strategies, also experimental based studies. However, the computational methods help in improved effectiveness in repurposing a drug. The computational methods help to select the effective candidate drugs before in-vitro-experiments [76]. Drug repurposing in breast cancer is considered an old weapon for new war. The immunotherapy approach in combination with chemotherapy is considered an important modality in TNBC treatment. As already discussed due to escape mechanisms in immunotherapy it is being combined with chemotherapy that repurposing the old school drugs for instance some FDA approved drugs like Anthracyclines and taxanes. Also these drugs are being repurposed to modulate the immune system response for better clinical outcome [74, 77]. For instance, cyclophosphamide that is an alkylating chemotherapeutic agent having well-built immunosuppressive activity and acts via cytotoxic or through immune enhancing mechanisms. However due to its high toxicity effects low-dose cyclophosphamide has been combined with immunotherapy options like immune checkpoint inhibitors, immune therapeutic agents including vaccines as well and it been tested and has shown better results in animal models [77]. Accordingly in this chapter we have provided a detailed account for the combination of immunotherapy with chemotherapy as an effective mechanism for drug repurposing that is using the different strategies to modulate existing drugs for efficient use.

13. Checkpoint inhibitors in combination with chemotherapy

In the process of immunotherapy, a combination with chemotherapy may be synergistic. Chemotherapy has been demonstrated to promote tumor cell antigen release, prompt class I MHC molecules, neoantigens, and expression of PD-L1, and stimulate activation of dendritic cells, which could improve the immune response release after or in the course of Immune Checkpoint Inhibitor treatment [78–80]. Combination therapies of checkpoint inhibitors and chemotherapy have showed significant results in TNBC. Pembrolizumab's safety profile and clinical efficacy have been examined in most of the analysis on inhibition of PD1 in TNBC. In highly positive PD-L1, untreated mTNBC patients who obtained pembrolizumab in conjunction with chemotherapy (PAX, nab-paclitaxel, carboplatin/gemcitabine), interim evaluation of the phase 3 KEYNOTE-355 (NCT02819518) trial shows a substantial increase in PFS (5.6 vs. 9.7 months) [81]. Pembrolizumab in combination with the microtubule inhibitor eribulin mesylate in the KEYNOTE-150/ENHANCE 1 (NCT02513472) trial showed a 25.6 percent ORR with an average progression free survival of 4.1 months [82]. The TONIC trial (NCT02499367) phase 2 analyzed the effectiveness of PD1 with nivolumab in previously treated mTNBC patients. The ORR for nivolumab treatment followed by doxorubicin was 35%, compared to 23% for CIS and 17% for patients who did not receive prior chemotherapy, implying that chemotherapy would cause an inflamed tumor microenvironment [83]. For LA or mTNBC patients treated with atezolizumab in conjunction with nab-paclitaxel, the clinical study GP28328 (NCT01633970) phase 1b showed an ORR of 39.4% and an average PFS of 5.5 months (**Table 4**) [84].

The first randomized Phase 3 trial to show the effectiveness of atezolizumab in conjunction with nab-paclitaxel in metastatic TNBC patients which were not treated previously was IMpassion130 (NCT02425891) [80]. The FDA and the European Medicines Agency (EMA) approved atezolizumab in conjunction with nab-paclitaxel as a primary trearment for PD-L1-positive, uneradicably, locally advanced, or mTNBC in 2019. The IMpassion131 trial (NCT03125902) phase 3 will

Trail id	Regimen	Disease setting	Phase	Recruitment status
NCT02819518	pembrolizumab + nab-paclitaxel or paclitaxel or gemcitabine/ carboplatin	Metastatic	III	Active, not recruiting
NCT02513472	pembrolizumab + eribulin mesylate	Metastatic	Ib	Active, not recruiting
NCT02499367	cyclophosphamide, cisplatin or doxorubicin followed by nivolumab	Metastatic	II	Active, not recruiting
NCT01633970	atezolizumab + nab-paclitaxel	Locally advanced, metastatic	I	Completed
NCT02425891	atezolizumab + nab-paclitaxel	Metastatic	III	Active, not recruiting
NCT03125902	atezolizumab + paclitaxel	Locally advanced, metastatic	III	Active, not recruiting
NCT03371017	atezolizumab + gemcitabine/ carboplatin or capecitabine	Locally advanced, metastatic	III	Recruiting
NCT02685059	neoadjuvant durvalumab + nab-paclitaxel + EC	early stage	II	Completed
NCT03281954	neoadjuvant atezolizumab + paclitaxel + carboplatin, followed by adjuvant atezolizumab + AC or EC	early stage	III	Recruiting
NCT03197935	neoadjuvant atezolizumab + nab-paclitaxel, followed by AC	early stage	III	Active, not recruiting

AC- doxorubin + cyclophosphamide; EC- epirubicin + cyclophosphamide.

Table 4.
Trials evaluating the use of immune checkpoint inhibitor in combination with chemotherapy.

assess the protection and effectiveness of atezolizumab in combination with PAX as a primary treatment in TNBC patients. The IMpassion 132 study (NCT03371017) examines the potential of previously treated, untreated, locally advanced and mTNBC patients who have not been eligible for the IMpassion130 trial may benefit from atezolizumab and chemotherapy (capecitabine, gemcitabine/carboplatin). Randomized study GeparNuevo (NCT02685059) phase 3 results demonstrated that durvalumab in conjunction with neoadjuvant chemotherapy based on taxane-anthracycline provides clinical benefits in early TNBC from 44% to 53% of pCR (pathological complete response) [85]. A neo-adjuvant chemotherapy (paclitaxel plus carboplatin) NSABP B-59 (NCT03281954) phase 3 is currently being recruited with atezolizumab, followed by atezolizumab adjuvant and chemotherapy. The Impassion031 (NCT03197935) trial, which combines atezolizumab neoadjuvent with concurrent nab-paclitaxel and chemotherapy based on anthracyclines in patients with an early stage TNBC, recently published interim results. Patients who were given atezolizumab in combination with chemotherapy had a pCR rate of 57.6%, compared to 41.1% in patients who obtained chemotherapy in combination with placebo [86].

14. Immune checkpoint blockade in combination with a targeted immunotherapy

14.1 Immune checkpoint inhibitors in combination with PARP inhibitors

Breast cancer patients with germline BRCA1 or BRCA2 mutations account for around 5% of all cases. While TNBC is the most common cancer with the mutation in BRCA1 gene, cancers linked to the BRCA2 mutation can turn up in any subtype of breast cancer with the same frequency as sporadic subtypes. Breast cancers with BRCA1/2 mutations have a deficiency in homologous recombination repair, a DNA double-strand break repair mechanism, the defect which has a lethal synergy with single-strand DNA repair inhibition [87]. The poly(ADP-ribose) polymerase (PARP) is involved in single-strand DNA repair, and PARP inhibitors have shown antitumor activity in patients with HER2-negative metastatic breast cancer who have BRCA1/2 germline mutations. The use of immune checkpoint inhibitors in combination with PARPi in TNBC patients has the ability to cause a powerful immune response against tumors due to the infiltrating T cell activation followed by tumor antigen release via PARPi-induced cell death. Moreover, PARPi has been shown to increase the expression of PD-L1 in cell lines, supplying additional support for combining treatment with checkpoint inhibitors [88].

The TOPACIO (NCT02657889) trail found that a combination of pembrolizumab with the PARPi niraparib resulted in an ORR of 29% in mTNBC patients [89]. The ORR was higher than what has been identified in similar patient populations for anti-PD1 monotherapy [64]. In addition, various clinical trials evaluating the PD-L1 inhibition combination with PARP inhibitors in mTNBC have been planned, two phase II studies included the combination of the PARPi olaparib with durvalumab (NCT03167619 and NCT03801369) and a phase II trial of atezolizumab in combination with olaparib (NCT02849496). In addition, triplet PD-L1 inhibition therapies with PARPi and VEGF inhibitors are currently being developed. A phase I/II analysis (NCT02484404) in case of progressive or recurring solid tumor is looking at the combination of durvalumab in conjunction with olaparib and cediranib the VEGFR inhibitor. According to preliminary findings, the recommended dosage was bearable and resulted in clinical benefit rate of 67% in 9 women having recurring solid tumors, TNBC was one of them (**Table 5**) [90].

Trail id	Intervention	Phase	Recruiting status
NCT02657889	pembrolizumab + niraparib	II	Active, not recruiting
NCT03167619	durvalumab + olaparib	II	Active, not recruiting
NCT03801369	durvalumab + olaparib	II	Recruiting
NCT02849496	atezolizumab + olaparib	II	Recruiting
NCT02484404	durvalumab + olaparib + VEGFRi	I/II	Recruiting
NCT02079636	Pembrolizumab+ Abemaciclib	I	Completed
NCT02322814	atezolizumab + taxanes + MEKi	II	Active, not recruiting

Table 5.
Combinations of PD1/PD-L1 antibody-targeted therapy in TNBC.

14.2 Immune checkpoint therapy and CDK4/6 (CDK4/6) inhibitors in combination therapy

In patients with ER-positive, HER2-negative metastatic breast cancers, pharmacological inhibitors of CDK4/6 have demonstrated remarkable activity [91–93]. Inhibitors of CDK4/6 have been demonstrated to improve anti-tumor immune response in preclinical models by manipulating two main immune evasion mechanisms in tumors [94–96]. First, CDK4/6 inhibitors elevate intracellular levels of double-stranded RNA by activating tumor cell expression of endogenous retroviral components. As a result, type III interferon synthesis is stimulated, which in turn improves tumor antigen presentation. Secondly, CDK4/6 inhibitors significantly reduce regulatory T-cell proliferation. Finally, these events facilitate tumor cell clearance by cytotoxic T cells, which can be intensified even further by the introduction of an immune checkpoint inhibitor. Abemaciclib in conjunction with pembrolizumab was studied in patients with HER2-, HR+, MBC in a phase I trial (JPBJ, NCT02079636). The main objective of the study was to determine the combination therapy's safety profile. A total of 28 patients were enrolled in the study. At the end of 24 weeks, four patients (14%) showed an analytical response. At the appropriate early time intervals in the MONARCH 1 analysis, this response was greater than the response shown by patients treated with abemaciclib monotherapy [97].

14.3 Combination of immune checkpoint inhibitors with MEK inhibitors

Suppression of the MAPK signaling pathway, which is frequently unregulated in TNBC and is correlated with enhanced proliferation of cells and shows resistance towards apoptosis, is another approach for combining immune checkpoint inhibitors with targeted therapy [98]. In the phase 2 COLET (NCT02322814) trial, cobimetinib the MEK1/2 inhibitor was combined with atezolizumab and PAX/nab-paclitaxel as a primary therapy in patients with LD or mTNBC. According to preliminary findings, paclitaxel in combination with nab-paclitaxel has a 34% ORR, while nab-paclitaxel has a 29% ORR [99]. Clinical studies of binimetinib the

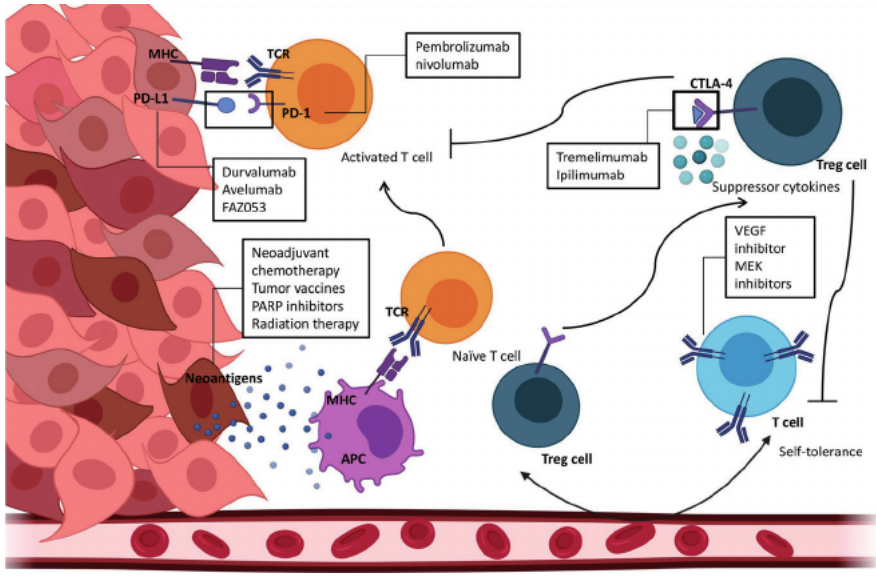

Figure 5.
Diagram representing the targets of immune checkpoint inhibitors.

Trail id	Intervention	Phase	Recruiting status
NCT03362060	pembrolizumab + PVX-410	I	Active, not recruiting
NCT02432963	pembrolizumab + p53-specific vaccine	I	Active, not recruiting
NCT03761914	pembrolizumab + WT1-specific vaccine	I/II	Recruiting
NCT03606967	durvalumab + Nab-paclitaxel+ neoantigen vaccine	II	Recruiting
NCT03199040	durvalumab + neoantigen DNA vaccine	I	Recruiting
NCT03289962	atezolizumab + neoantigen vaccine	I	Recruiting

Table 6.
Current clinical trials for cancer vaccine and immunotherapy.

MEK inhibitor in conjunction with pembrolizumab (NCT03106415) or avelumab (NCT03971409) in patients with LD or mTNBC are also underway (**Figure 5**).

14.4 Combination therapy: PD1/PD-L1 antibody and cancer vaccine

Cancer vaccines are a novel approach to cancer immunotherapy. These vaccines promote T cell priming and activation and strengthen immune recognition of cancer cells by presenting breast cancer peptides to T cells. Monovalent vaccines, which provide a single tumor-associated antigen (TAA) target for the immune system, and polyvalent peptide vaccines, which provide several TAA targets, are two types of cancer vaccines. Low response rates have hampered the application of peptide vaccines for the treatment of patients with metastatic cancer; although, making use of a multi-peptide vaccine strategy, the response rate in various cancer types has improved to 9.9% [100, 101]. Furthermore, cancer vaccines in conjunction with immune checkpoint inhibitors can improve the vaccine's anti-tumor immune response. In advanced TNBC, a few ongoing studies are looking into the effectiveness of cancer vaccines in conjunction with pembrolizumab, making use of either the multipeptide vaccine PVX-410 (NCT03362060) or specific vaccines which target p53 (NCT02432963) or WT1 (NCT03761914). Furthermore, few clinical trials have been conducted to investigate the efficacy of durvalumab in combination with the multipeptide vaccine PVX-410 (NCT02826434) or with a neoantigen vaccine (NCT03606967, NCT03199040), as well as atezolizumab in combination with a neoantigen vaccine (NCT03289962) (**Table 6**).

15. Combining immunotherapy with epigenetics in cancer treatment

Immunotherapy arguably is one of the exciting new developments for the management of advanced human tumors, in particular the concept of immune checkpoint blockade [102–104]. Antibodies targeting PD-1, CTLA-4 and PD-L1 show robust responses in treatment of melanoma, and in high grade tumors. Although, these recent advances are very exciting and promising, however majority of the tumor patients including TNBC patients show little or no response at all to immune checkpoint therapy alone [105, 106].

Therefore raising an apparent question as to whether immunotherapy could work in combination with other therapies like immune checkpoint targeting agents to enhance the clinical response and efficiency of various sub types of cancers. Nevertheless, various clinical trials as like previously discussed are evolving while keeping in control the related toxicities [107].

Other combination strategies targeting immunotherapy in combination with chemotherapy as well targeted therapy approaches likely epigenetic therapy. As epigenetic therapy has been evidenced to strongly sensitize patients to immune checkpoint therapy.

16. Definition of epigenetic therapy

The term epigenetic therapy is now widely used, and involves use of drugs or other epigenome-influencing mechanisms for treatment of human disorders. Recent advances have delineated regulatory mechanisms of the cancer and normal epigenomes and the functional understanding of histone modifications, methylation patterns, and dynamics of nucleosomes [108, 109]. Recent studies in the field of cancer epigenetics have not only defined key targets for cancer management but also provided key insights in drug repurposing for modulating cancer epigenomes [110]. In epigenetic therapy, drugs target three specific protein categories (a) Writers, enzymes that establish epigenetic marks; (b) Readers, proteins that recognize histone and may bring in other protein complexes to change gene expression; and (c) Erasers, enzymes that remove epigenetic marks [111]. Drugs that impede writers of DNA methylation, DNA methyltransferases (DNMT), and erasers (histone deacetylases or HDAC) that regulate histone lysine acetylation are central to epigenetic therapy in cancer treatment. HDACs and DNMTs are mostly linked with transcriptional repression. Thus, inhibiting HDACs and DNMTs can upregulate expression of involved genes with many consequences for downstream pathways of this gene activation.

Cytidine analogues inhibit DNMTs by blocking their catalytic and likewise induces their degradation [112]. Also, the degradation of DNMTs can remove key scaffolding properties that may function for repression of transcription [113, 114]. Tumors show significant alterations in DNA methylation of cytosines at CpG dinucleotides such as loss of methylation at regions such as repetitive elements that must be silenced for genome stability and gain of methylation at the promoter regions of tumor suppressor and other genes [115]. Inhibitors targeting DNMTs promote reactivation of tumor suppressor, silenced by promoter DNA methylation [116]. DNA methylase inhibitors (DNMTi) showed augmented apoptosis, decreased cell cycle activity, and reduced stemness in a transient exposure to several cancer cells (**Figure 6**) [117]. DNMTis such as 5-azacytidine and 5-aza-20-deoxycytidine showed robust efficacy in treatment of hematological disorders and has been approved by FDA for the treatment of myelodysplastic syndrome (MDS) [118]. Several clinical studies are undergoing presently to study the effect of epigenetic therapy in cancer treatment **Table 7**.

Histone modifications by acetylation plays a central role in epigenetic gene regulation by altering the condensation status of chromatin, modulating the accessibility of transcription factors to target DNA sites. Histone acetyltransferases (HAT) and HDACs maintain the acetylation state of histones of nucleosomes. Inhibitors targeting HDACs known as (HDACi) are presently approved for the treatment of peripheral T-cell lymphoma (PTCL) and cutaneous T-cell lymphoma (CTCL), although it is yet to be known as why these two cancers are highly sensitive towards HDACi [119, 120]. Also, it has been observed that HDACi show dependency of, compound, dose and pleotropic characteristics. Many of the HDACi directly affect acetylation of histone proteins and modulate epigenetic changes while some affect acetylation of non-histone or cytoplasmic proteins [121]. Besides, it has been observed that transient exposure of tumor cells to low doses of DNMTs, followed by HDACi treatment increases gene expression of hypermethylated genes.

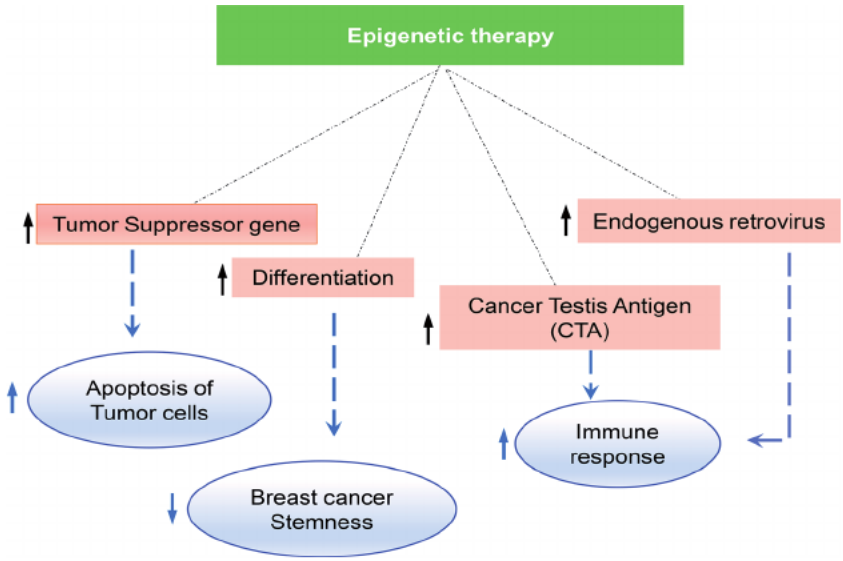

Figure 6.
Flowchart representing the overall effects of epigenetic therapy.

Epigenetic inhibitor	Target	Type of cancer
Entinostat	HDAC1/HDAC3	Recurrent or refractory solid tumors
KA2507	HDAC6	Solid tumors
Tazemetostat	EZH2	Advanced solid Tumors
AZD5153	BRD4	Advanced solid tumors and lymphomas
Triple: Entinostat, Nivolumab and Ipilimumab	HDAC/ICB	Locally advanced or metastatic HER2-negative breast cancer
Entinostat plus Pembrolizumab	HDAC/ICB	Advanced solid Tumors
CC-486 plus Durvalumab	HMA/ICB	Colorectal, ovarian, and breast tumors
CPI-1205 plus Ipilimumab	EZH2/ICB	Advanced solid tumors

Table 7.
Clinical trials for epigenetic inhibitors.

17. Connecting epigenetic modulation with immunotherapy

Over In the past two decades, the FDA approval of various DNA methyltransferase inhibitors, collectively called DNA HMAs, and histone deacetylase inhibitors (HDACi) has brought epigenetic therapy to the forefront of cancer therapies. However, the benefits of epigenetic therapy are mainly restricted to the treatment of hematological malignancies. Thus, combination strategies with standard chemotherapy and targeted therapy approaches can be considered. A recent study involving advanced NSCLC patients revealed that patients after receiving low-dose epigenetic therapy entered a trial for immune checkpoint therapy. Approximately 20% of the patients responded to the immune checkpoint therapy alone, passing 24 weeks without progression, with most achieving high-grade RECIST criteria responses [122]. This is an astounding result for immunotherapy in NSCLC.

All 5 patients who had received the prior epigenetic therapy passed the 24-week point without progression with subsequent immune checkpoint therapy and three of these developed high-grade partial RECIST criteria responses that have all been durable over 2.5 years [123, 124]. Moreover, findings to date, support the hypothesis that there may be extraordinary potential for combined epigenetic and immunotherapy to increase the frequency of durable responses for immune checkpoint therapy in not only NSCLC but also other common tumor types.

18. Epigenetic therapy drugs boost immune attraction properties of epithelial cancer cells

Immunotherapy has presently become a remarkable tool to employ immune cells in tumor management. Blocking immune checkpoints to stimulate and retore immune response in the tumor immune suppressive microenvironment has showed robust clinical response. However, several patients tend to remain unresponsive towards immune checkpoints blockades. Epigenetic therapy using DNMTis and HDACis have showed potential in immune modulation properties of tumor cells and immune cells, thereby suggesting a rationale for integrating epigenetic with immunotherapy.

It is well known that cytotoxic T cells (Tc) are requisite for an anti-cancer immune response and immune check point blockade. This mechanism relies on antigen presenting cells and the quantity of antigens presented to Tc cells. Also, tumors with high mutations show robust response to immune check point blockade due to high presence of neo-antigens presented to Tc cells [125, 126]. Several studies demonstrate that high immunogenicity is followed by exposure to epigenetic therapy. DNTMis have been found to upregulate and augment expression of cancer testis antigens (CTAs) such as MAGE-A1 and NY-ESO-1 [127]. Besides, exposure to epigenetic therapy viz. HDACis and DNMTis also upregulated antigen presenting and processing related genes such as b2-microglobulin, Human leukocyte antigen (HLA)-class I genes, and TAP1 in solid tumors [128, 129]. Furthermore, it was revealed that HDAC inhibitors stimulate human endogenous retroviruses (HERVs) reactivation, which induce activation of pattern recognition receptors and a type I/III interferon response thereby enhancing antigen presentation to Tc cells [129, 130]. Together, these results paint the picture that epigenetic therapy using HDACis and DNMTis augment presentation of CTA and HERV-derived antigens, thus enhancing immune response in low mutation therapy [131]. In AML patients, epigenetic therapy with DNMTis promoted robust T cell mediated immune response by reactivation of CTAs [132]. The host immune system recognizes the CTAs with high affinity, they represent good candidates for immunotherapy, including vaccines. There is thus great potential for DNMT inhibitor treatment to upregulate CTAs on tumors, facilitating targeting by the host immune system [133]. Guo et al. demonstrated that exposure of 4T1 mammary carcinoma cells in syngeneic mice to DNMTi 5-aza-2-deoxycytidine induced demethylation and upregulation of CTA P1A. Also, the upregulated P1A was targeted by P1A–specific T cells, and combined therapy with 5- aza-20-deoxycytidine and adoptive transfer of these T cells significantly reduced lung metastases in this mouse model [134].

Additionally, synergistic relation was observed in pre-clinical models of diffuse large B cell lymphomas for combinatorial exposure to DNMTis and HDACis [135]. Increasing evidence suggests that tumors possess variable numbers of infiltrated immune cells and the quantity, type, and location of infiltration can help in predicting response to immune check point blockade [36, 136]. It is now well established that epigenetic therapy with modulates directly infiltration of immune cells in

tumor stroma. DNMTi treatment in addition to inhibiting tumor progression, increased infiltration of CD8+ T cell infiltration, and natural killer (NK) cells and reduced infiltration of immune-suppressive cells [131, 137]. Also, HDACis treatment in combination with DNMTis activates chemokine signaling networks and augments infiltration of cytotoxic T cells [138]. In preclinical studies, treatment with romidepsin, the pan-HDAC inhibitor, augmented expression of chemokines by tumor cells which elevated infiltration of T cells into the tumor stroma and reduced tumor growth by robust immune response [139].

Accumulating evidence from preclinical models of diverse solid tumors viz. breast, melanoma and colorectal cancer, revealed that combining immune check point inhibitors such as anti-CTLA4 or anti-PD1 with epigenetic therapy (DNMTis and HDACis) augmented antitumor response and reduced tumor growth and response to immunotherapy than using monotherapy of either agent [122, 136]. Also, combinational treatment with DNMTis and anti-CTLA4 antibody enhanced chemokine expression and increased survival of mice with orthotopic or subcutaneous tumors [137].

Together, these results paint the picture that combining immunotherapy with combinational therapy, greatly enhances antitumor immune responses, by augmented expression of chemokines and these act in a synergistic manner. Also, multiple clinical trials are currently testing the combination of DNMTi or HDACi with various immune check point inhibitors (**Table 7**).

19. Integrating immunotherapy with oncolytic viruses for cancer treatment

The antitumor activity of oncolytic viruses involves multiple mechanisms that encompass the natural interactions between viruses, tumor cells and the immune system [140]. During the last decade oncolytic viruses are becoming an effective means in cancer treatment. Viruses have developed sophisticated means to escape immune surveillance and which can be manipulated for therapeutic purposes to stimulate anti-cancer immune response. Likewise, nearby infusion of oncolytic virus into a tumor site can incite an abscopal impact, in which distant, uninfected tumors additionally go through insusceptible immune rejection [141]. This abscopal effect is caused by oncolytic viruses' sequential activity, multiply in cancer cells and then progresses to activation of immunogenic cell death, which results in the release of antigens and danger factors, which then enhance both innate and adaptive antitumor immune responses. Furthermore, oncolytic viruses can be genetically modified to express therapeutic genes, which can improve antitumor activity even more. In the absence of viral replication, viral encoded gene expression allows immune regulation against tumors while restricting the antiviral immune response [142]. This points out, oncolytic viruses are highly adaptable agents that offer a critical 'on' switch that enhances the migration of tumor infiltrating lymphocytes into the tumor stroma, and this can be exploited to improve antigen-specific immune responses as part of combo-immuno therapies.

20. Characteristics of oncolytic viruses

Viruses are microscopic particles that selectively replicate in the interior milieu of host cells, and inflammation and underlying pathogenicity can be associated with viral infection [143]. During the last decades, viruses have been employed in delivery of therapeutic genes for the treatment of metabolic and degenerative

illnesses, immunization against infectious diseases, and as oncolytic agents for cancer therapy [140].

The genome, which is either single-stranded or double-stranded RNA or DNA; the capsid, which is a protein coat that covers the genetic material; and the capsid, which is a protein coat that covers the genetic material, also in certain viruses, the lipidic envelope which surrounds the capsid and may enhance virus adhesion to host cell membranes, so increasing viral penetration, are the three major structural parts of most viruses. Oncolytic viruses have been developed over the last decade using both DNA and RNA viruses. DNA viruses offer several advantages: their huge genomes can be altered without interfering with viral replication; big eukaryotic transgenes may be incorporated by DNA viruses to boost therapeutic effectiveness or immunological regulation; DNA viruses express high fidelity DNA polymerases, assuring viral genome integrity and effective replication; and there is little, if any, nuclear integration of DNA viruses **Table 8** [144]. RNA viruses offer additional advantages: because they are smaller than DNA viruses, they can pass the blood–brain barrier, allowing tumors in the central nervous system to be targeted [145]. Despite the fact that their short genome restricts their capacity to encode big transgenes, because pre-existing tolerance to certain RNA viruses is poor in humans, viruses are more suited for systemic distribution, at least for the brief period before antiviral immunity is generated. Furthermore, the detection of viral double-stranded RNA by protein kinase R (PKR) that happens in normal cells may not occur in tumor cells, which often have lower levels and phosphorylation of PKR [146, 147]. Many aspects influence the selection of oncolytic viruses for tumor immunotherapy, in particular high pathogenicity, immunogenicity, cancer tropism, the potential to encode therapeutic transgenes, feasible viral concentration during synthesis, and durability. The active phase of viral infection and reproduction in host cells is described by the lytic virus life cycle [148]. Attachment, penetration and uncoating, synthesis, assembly, and release are the five different phases of the viral life cycle, which may be managed by genetic modification of the viral genome and can serve as a physiologically realistic strategy for selectively targeting tumor

	Adenovirus	**Coxsackie virus**	**Maraba virus**	**Pox virus**
Genome	dsDNA	ssRNA	ss (−) RNA	dsDNA
Genome size	Moderate (32 kb)	Small (~8 kb)	Small (11–15 kb)	Large (130–375 kb)
Cell entry mechanism	Endocytosis	Micropinocytosis via epithelial tight junctions	Endocytosis; pH dependent fusion activation	Membrane penetration and fusion
Cell entry receptors	hCAR VCAM1 CD46	CAR DAF	Unknown	GAGs EFC
Transgene capacity	Moderate	Low	Very low	High
Viral immunogenicity	Low	Low	Low	High
Ability to penetrate Blood brain barrier	Very limited	Moderate	Limited	Very limited

Table 8.
Characteristics of oncolytic virus.

cells for infection and viral replication. Viruses also display pathogenicity and immunogenicity, which vary depending on viral species, dosage, mode of administration, pre-existing host immunity, and other variables, and are characteristics that can produce effective antitumor immunity.

21. Anti-tumor activity of oncolytic viruses

Considering they influence multiple crucial phases in the cancer–immunity process, oncolytic viruses offer several benefits as cancer treatment agents [149]. These features include preferential replication in tumor cells, stimulation of immunogenic cell death and release of soluble antigens and danger signals, induction of innate immune responses by recruitment of immature dendritic cells (DCs) and innate lymphoid cells, correction of antigen processing and presentation abnormalities, and activation of adaptive immunological responses. Although the molecular and cellular intricacies of how oncolytic viruses correct these processes are not entirely known, advances in the generation of antitumor immunity employing oncolytic viruses are being achieved, and insights into rational combination therapy based on oncolytic viruses are being explored.

22. Combing oncolytic virus treatment with immune check point blockade

Immune check point blockade therapy (ICB) is extensively in cancer treatment, and long-term clinical outcomes are promising. Clinical responses are associated with pre-existing antitumor immune responses, such as an increased number of TILs, a high mutation load, and the formation of a diverse neoantigen repertoire [150, 151]. Combination therapy utilizing ICB and oncolytic viruses are appealing because the oncolytic virus can drive recruitment of TILs into immune-deficient tumors and prompt the production of soluble tumor antigens, danger signals, and pro-inflammatory cytokines, which can improve T cell recruitment and boost immune cell activation. Viral infection also raises the expression of CTLA4, PDL1, and other immunological checkpoint molecules, which would normally inhibit T cell activation (and so antitumor immunity), but also makes tumors more susceptible to ICB (**Figure 7**) [152, 153]. Preclinical research with a B16–F10 melanoma indicated that localized injection of tumors with oncolytic Newcastle disease virus caused infiltration of tumor-specific CD4+ T cells and CD8+ T cells into both the injected tumor and distant tumors, as well as improved tumor susceptibility to systemic CTLA4 inhibition 18. An oncolytic virus Maraba demonstrated therapeutic potential as a neoadjuvant in a preclinical model of triple-negative breast cancer and sensitized previously refractory tumors to ICB [154]. Several additional oncolytic viruses, including B18R-deficient vaccinia virus and vesicular stomatitis virus expressing a library of melanoma antigens (VSV- ASMEL), also shown substantial (P 0.05) therapeutic effect when used in tandem with ICB [155, 156]. Administration of T- VEC intratumorally, followed by anti-CTLA4 antibody (ipilimumab) treatment via intravenous injection, demonstrated an object response rate of 50%, with 44% of patients showing robust responses lasting more than 6 months in a phase Ib clinical trial. Also, no dose limiting toxicities were observed in the patients [157]. Additionally, a recent study reported that treatment with oncolytic poxvirus CF33-hNIS-ΔF14.5 modulates tumor microenvironment in TNBC model, and increases the response of tumor cells towards anti-PD-L1 antibody. Tumor microenvironment is one of the central plays in tumor growth, metastasis and

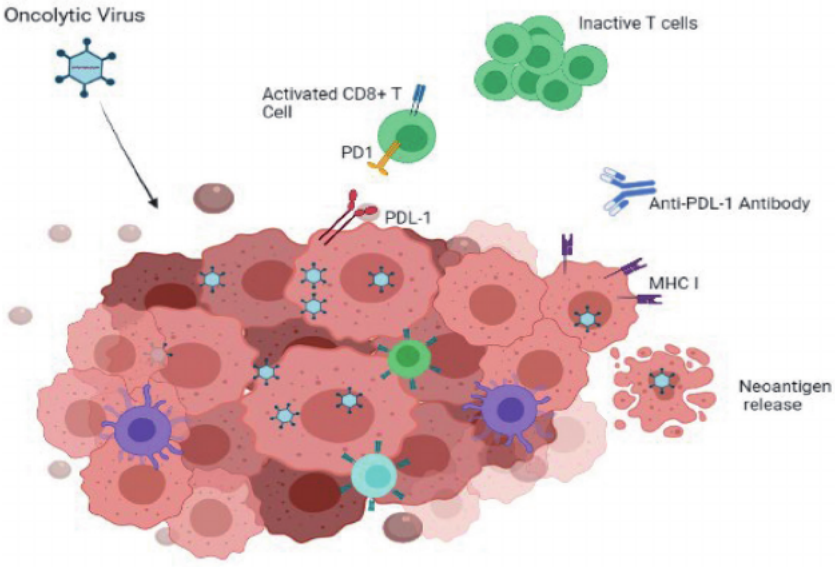

Figure 7.
Represents the role of oncolytic virus in immunotherapy.

development of resistance. Further *in vivo* and *in vitro* analysis revealed that infection with the virus stimulated expression of PD-L1 in TNBC cells. Also, exposure of mice model of TNBC to oncolytic poxvirus CF33-hNIS-ΔF14.5 enhanced infiltration of CD8+ T cells and increased expression of proinflammatory cytokines IFNγ and IL-6 by tumor cells. Combinational treatment with oncolytic poxvirus CF33-hNIS-ΔF14.5 and anti-PD-L1 antibody augmented TME modulation and induced 50% tumor regression in mice models. Administration of these as single agents failed to inhibit tumor growth. Besides, it was also observed that the recovered mice with combinational treatment did not develop tumor after re-challenge with the same cancer cells suggesting that they developed immunity against those cancer cells [158, 159].

Taken together, studies demonstrate that oncolytic virus treatment positively induces tumor immune microenvironment modulation in triple-negative breast cancer model making them responsive to the immune checkpoint inhibitors and hence warrants further studies to determine the clinical applicability of this combination approach.

23. Summary

1. Chemotherapy lacks the success in treating malignant tumors like TNBC as it lacks specificity and can act on normal healthy cells causing secondary diseases in patients.

2. Furthermore, immunotherapy have shown downfall in its efficacy due to the major problem of escape of tumor cells from the immune response against them.

3. Therefore, drug repurposing a strategy commonly used to reprofile or repurpose the existing chemotherapeutic drug has shown promising effects in targeting various diseases including malignant tumors.

4. Drug repurposing is mainly done by using both computational and non-computational methods including target-based computational studies and in vitro based experimental studies

5. These methods permit us to select an existing drug whether FDA approved or drugs that are under investigational studies before in vitro studies thus reducing time consumption and proving cost effective.

6. Because most chemotherapeutic drugs are toxic in nature and lack target specificity as well, therefore by using drug repurposing approach we can combine the chemotherapeutic drugs with target specific immunotherapeutic options to make them effective.

7. Therefore, chemotherapeutic drugs can be combined with immune checkpoint inhibitors, PD-1/PD-L1 antibody and vaccines to provide promising results in anti-tumor response

8. Various enlisted clinical trials have shown promising results in combining chemotherapy with immunotherapy.

24. Future perspective

TNBC is the most aggressive, lethal and complex subtype of breast cancer. What makes it more aggressive is the lack of targeted therapies leaving chemotherapy as the main treatment option available. However, chemotherapy itself mostly lacks target specificity and can harm normal healthy cells of an individual. Moreover, another treatment option that is immunotherapy also faces some problems showing inefficacy due to escape of tumor cells from immune surveillance. Nevertheless, a strategy known as drug repurposing has shown to be a promising strategy to overcome the inefficacy of available treatment options. In drug repurposing, an existing chemotherapeutic drug can be repurposed to modulate its efficacy. In this chapter, we have focused primarily on repurposing the available drugs whether PARP inhibitors or MEK inhibitors, vaccines including the ones under clinical trials as well by combining them with other available immunotherapeutic options like immune checkpoint inhibitors, PD-1/PD-L1 antibodies etc. Also the currently used epigenetic therapy drugs also are known to show significant efficacy in modulating immunotherapy responses in patients suffering from cancers especially TNBC. From our point of view combining drugs with other target specific drugs like drugs targeting immune system components provides a significant insight as it repurposes the drug whether chemotherapeutic or epigenetic drug making it target specific.

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Role of Activated Cdc42-Associated Kinase 1 (ACK1/TNK2)-Inhibitors in Precision Oncology

Ruby Srivastava

Abstract

Activated Cdc42-associated kinase 1 (ACK1) is an intracellular non-receptor tyrosine kinase referred to as TNK2, which is considered as an oncogene and therapeutic target in various cancers including breast cancer, non-small-cell lung cancer (NSCLC), hepatocellular carcinoma (HCC), and many others. Oncogenic non-receptor tyrosine kinase mutations occur either due to point mutations, duplications or insertions and deletions, or by involving in the development of a fusion gene resulting from a chromosomal rearrangement. ACK1 is involved with multiple signaling pathways of tumor progression. With these signaling networks, ACK1 participates in cell survival, invasion, migration, and tumorigenesis that are strongly related to the prognosis and clinicopathology of cancers. Previous studies predicted that ACK1 is a carcinogenic factor and blockage of ACK1 inhibits cancer cell survival, proliferation, migration, and radiation resistance. FDA has approved many multi-kinase inhibitors as therapeutic drugs that show good inhibitory activity not against ACK1 but also towards multiple targets. As ACK1 is a key target for other neurological diseases, inflammation, and immunological diseases also, so the studies on these inhibitors not only provide potential strategies for the treatment of cancers that require simultaneous targeting of multiple targets but also can be used in drug repurposing for other diseases.

Keywords: inhibitors, therapeutics, signaling pathway, prognosis, clinicopathology

1. Introduction

Tyrosine kinases are enzyme family member which interpose the movement of the phosphate group to tyrosine residues of target protein, thus transmitting signals from the cell surface to cytoplasmic proteins and the nucleus to regulate physi-ological processes. TKs are divided in two sub groups: receptor and non-receptor proteins. Receptor tyrosine kinases (RTKs) include Platelet-derived growth fac-tor receptors (PDGFR), Fibroblast growth factor receptor (FGFR), Epidermal growth factor receptor (EGFR), and Insulin receptor (IR). The Non-receptor TKs (NRTK) are divided in 9 sub-families based on the sequence similarities which included Abl, FES, JAK, ACK, SYK, TEC, FAK, SRC, and CSK. Activated Cdc42-associated kinase 1 (ACK1/TNK2) (PDB code-6VQM) is a non-receptor tyrosine kinase, which belongs to VIII tyrosine kinase family. There are seven different types of ACKs as, ACK1/TNK2, ACK2, DACK, TNK1, ARK1, DPR2 and KOS1 [1].

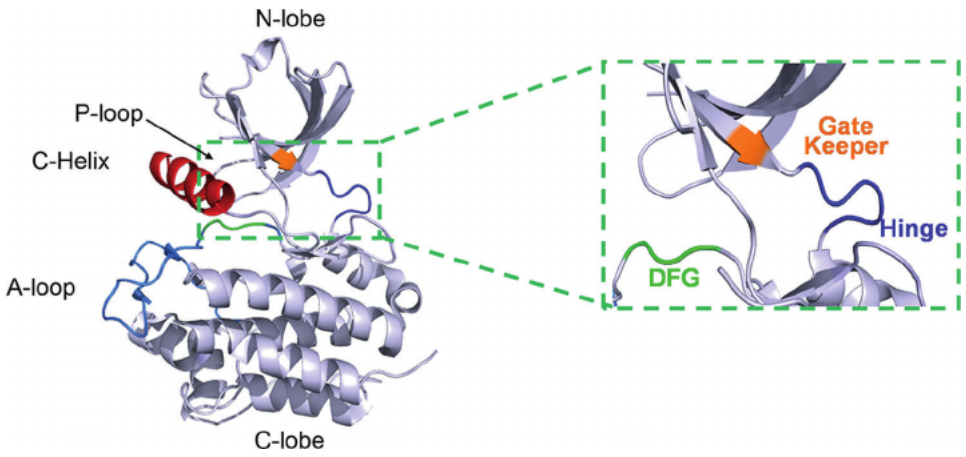

Figure 1.
The crystal structure of ACK1/TNK2 protein with loop (A, C), lobe (C, N) and helix (C) with PDB code-6VQM. The DLG, gate keeper and hinge is represented in a separate box. Adapted from Aoxue Wang et al. [6].

ACK1 was identified as first effector protein of Cdc42 [2, 3], and was cloned in hippocampus of the human brain that binds to GTP-bound form of Cdc42 [4] and inhibits its GTPase activity. ACK regulates about 147 proteins expression which is strongly connected with cell survival mechanisms [5]. The crystal structure of ACK1 is given in **Figure 1**.

ACK1 is an approximately 114 kDa protein and have 1038 amino acids. ACK1 consists of 8 domains; sterile α motif domain (SAM), tyrosine kinase domain (TKD), Src homology 3 domain (SH3), Cdc42/Rac-interactive binding motif (CRIB), clathrin-binding region (CLATH), PPXY motif or WW domain-interacting region, epidermal growth factor receptor-binding domain (EBD) or Mig-6-homology region (MHR), and ubiquitin association domain (UBA). The SAM domain is related to membrane localization, dimerization, and activation of ACK1 (**Figure 2**) [7].

Its coding gene TNK2 is located on 3q29. The main function of TNK2 is to regulate the cell cycle by binding to CDC42 [8]. TNK2 can also act as an effector of CDC42 to regulate cellular attachment and migration [9]. The CRIB domain is important for ACK1 activation and its cytoskeletal functions. ACK1 is more specified for Cdc42 activation over other GTPases (Rac and Rho) [10]. The second half of ACK1 has GRB2 [2], Sortin nexin 9 (SNX9) [10], and cortactin [11] as SH3 domain-containing binding partners. The frequent amplification and mutations of ACK1 leads to the abnormal activity of the ACK1 signaling cascades [12]. TNK2 is related to the hematological malignancies and other types of cancers [5, 13–16]. The structure of ACK family includes ACK1, 38-negative kinase 1 (TNK1), their splicing variants, activated Cdc42-associated kinase 2 (ACK2), kinase of embryonic stem cells (Kos1), and homologous proteins. It can be easily identified in mice, cows and fruit flies (ACK (Dack)) and A Ras-regulating kinase 1 (Ark-1). TNK1 is the first tyrosine kinase in which the tumor suppressor activity is found. TNK1 participates in inflammatory responses and promotes apoptosis. Its genetic variation is related to the Alzheimer's disease. ACK1 has a special structure, which gives it unique regulatory functions. ACK1 can integrate many RTK signals and proved to be associated with cancer cell survival, proliferation, migration, and radiation resistance. It is used for cancer prediction and prognosis also. The multidomain structure of ACK1 has ability to bind to a variety of proteins, which is not only conductive to the precise location of ACK1, but also promotes its various diversified functions.

ACK1 act as an important transducer of variety of extracellular signals [11]. The amplification of ACK1 gene can cause ACK1 phosphorylation (p-ACK1) and

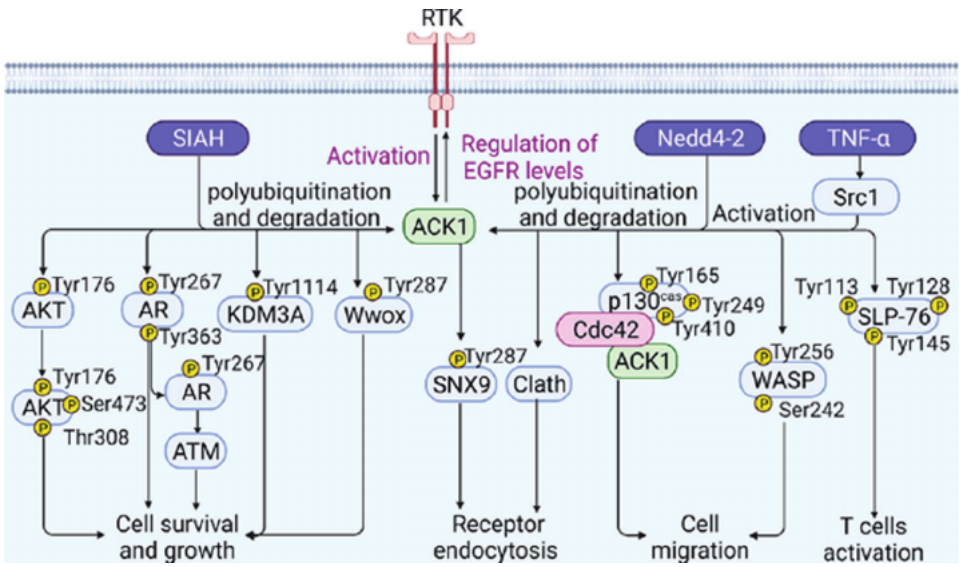

Figure 2.
Representation of downstream signaling pathways of ACK1. The full names of these kinase proteins are ACK1 (activated Cdc42-associated kinase 1); AKT (protein kinase B); AR (androgen receptor); ATM (ataxia telangiectasia mutated); Cdc42 (cell division cycle protein 42); KDM3A (lysine (K)-specific demethylase 3A); p130Cas (p130 Crk-associated substance); RTK (receptor tyrosine kinase); SIAH (seven in absentia homolog); SLP-76 (SRC homology 2 domain-containing leukocyte phosphoprotein of 76 kDa); SNX9 (sorting nexin-9); SRC1 (steroid receptor coactivator 1); TNF-α (tumor necrosis factor α); WASP (Wiskott– Aldrich syndrome protein) and Wwox (WW domain-containing oxidoreductase). Adapted from Aoxue Wang et al. [6].

auto-activation, which results in the activation of ACK1 signal transduction [15, 17]. Activated ACK1 senses extracellular signals while interacting with activated receptor-tyrosine kinases including AKT, EGFR, HER2 and MERTK [18], clathrin, WW domain-containing oxidoreductase (Wwox), Grb2, AKT1, ubiquitin, androgen receptor, and Nedd4-1/2 E3 ligases [19–23]. Further studies indicated that tyrosine kinases directly regulate the activity of DNA repair and cell cycle check point proteins by tyrosine phosphorylation. ACK1 as an oncoprotein which act as an epigenetic regulator. Tyrosine kinases epigenetically regulate DNA damage signaling pathways by modifying the core histones as well as chromatin modifiers at critical tyrosine residues. The deregulated tyrosine kinase driven epigenomic alterations have intense inferences in malignancies, aging and genetic abnormalities (**Figure 3**).

ACK1 phosphorylates and activates key survival-promoting kinase receptors on different tyrosine residues and eliminates tumor suppressors through similar mechanisms, resulting in cell survival, proliferation, and migration. ACK1 can interact with several components of vesicle dynamics in cell endocytosis and trafficking. ACK1 plays an important role in promoting extrinsic apoptosis, intervene in mechanically-induced inhibition of growth and weaken mitogenic signals to avert the abnormal growth of tissues.

The physiological roles of ACK1 include both the cancer and the normal tissues. In cancer, ACK1 participates in the regulation of many signaling pathways and exerts corresponding physiological functions, which include proliferation, differentiation, survival, apoptosis, migration, and epidermal-mesenchymal transition (EMT) and influences several important cellular processes. ACK1 is frequently overexpressed in various aggressive tumors also. It was found that ACK1 is a molecular component of the signaling cascade of neurotrophins. It is highly expressed in human brain and plays important physiological function in inflammation and immune system.

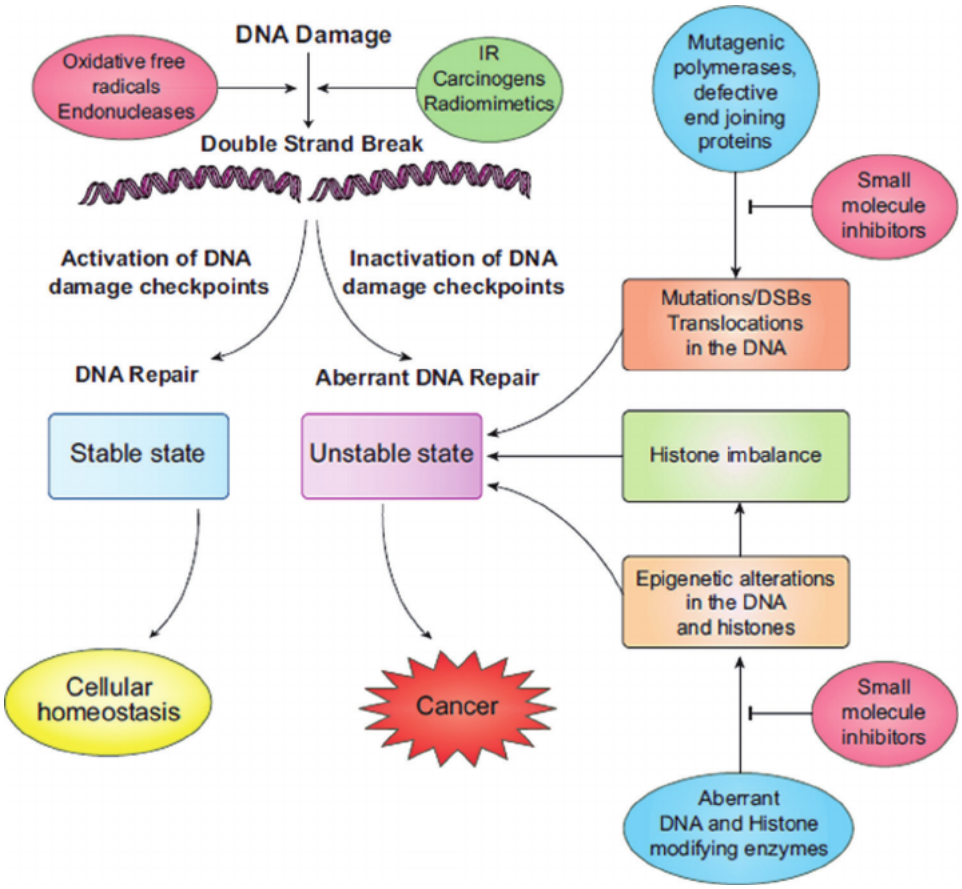

Figure 3.
Representation of various exogenous and endogenous agents activating DNA damage checkpoints in cancers. Chromatin alterations can also activate DNA damage signaling pathways. Activated checkpoint kinases, ATM or ATR arrest the cells at a specific stage in the cell cycle and allow time for repair. DNA double strand breaks due to ionizing radiation may be repaired either by the homologous recombinational repair pathway (HRR) or the non-homologous end joining pathway (NHEJ). Eukaryotic cells face a many situations, which lead to unstable genomic states, aberrant activity of the end joining proteins and mutations in the DNA and histone modifying enzymes. Small molecule inhibitors can be a therapeutic option to restore genome stability and also inhibiting tumor growth by radio sensitization. Adapted from Mahajan and Mahajan [24].

There are three ways to activate ACK1, which are RTK interaction, somatic cell missense mutation, and gene amplification. In previous studies, mutations in ACK1 genes have been observed in 21 kinds of cancers. 131 missense mutations, 39 nonsense mutations, and 3 fusion mutations are found in different regions of ACK1 [6]. The gene amplification of ACK1 is also observed in approximately 20 types of cancers. In cancers ACK1 is a key drug target of approximately 24 types of cancers as Metastatic Colorectal Cancer, Breast Cancer, Leukemia, Prostate Cancer, Melanoma, Gastric cancer, Lung cancer and many more. In one of RNA sequencing studies on Non-small Cell lung cancer (NSCLC) it was found that silencing of ACK1 upregulated several immune pathways as T cell receptor signaling, PI3K-Akt, Ras signaling pathways, MAPK, cAMP, Wnt signaling pathways. It was observed that ACK1 gene copy numbers were inversely linked with the infiltration levels of B cell, CD8+ T cell, CD4+ T cell, macrophage, neutrophil, and dendritic cells in NSCLC [25]. Studies showed that many ACK1 tyrosine kinase signaling proteins in many tumor cells are activated repeatedly in breast cancers and the expression of ACK1 is positively correlated to the disease

severity progression and negatively correlated to the survival rate in breast cancer patients [4, 12, 26–30]. However clinical trials of targeting ACK1 in triple negative breast cancers (TNBCs) have not shown any promising results with specific inhibitors. Many tyrosine kinases (EGFR), oncoproteins (AKT), tumor suppressor proteins (Wwox), and epigenetic modification regulatory proteins (KDM3A) interacts with ACK1 in breast cancer [4, 28–32]. The clinical trials in hepatocellular carcinoma (HCC) studies predicted that ACK1 was highly expressed to the HCC tissues than in non-HCC tissues and further analysis indicated that ACK1 is positively correlated with p-ACK1 and negatively correlated with WWOX expression in HCC. The investigation revealed that ACK1 can act as potential prognostic biomarker and therapeutic target in HCC [33]. TNK2 and miR-125a-3p are considered as potential diagnostic and therapeutic targets in Colon cancer [34]. TNK2 drives the malignant state via a feed-forward ACK1/pY88-H4/WDR5/MLL2/AR epigenetic circuit in castration-resistant prostate cancers [35] and prostate cancer survival [36].

As ACK1 is highly expressed in many cancers and play a major role in tumor occurence, targeting ACK1 gives a promising strategy for tumor treatment. Interestingly, increased Cdc42-dependent Ack1 phosphorylation has been observed in cells depleted of dynamin, and in these cells, ACK1 showed enhanced binding of both endocytic and ubiquitylated proteins [37]. ACK1 has shown potential to overcome drug resistance and provide novel possibilities of drug combination schemes for targeted therapies in cancer treatment. Kinase Inhibitors as a major drug class were emerged after the FDA approval of imtinib in 2001. Till now there are 71 small-molecule FDA approved kinase inhibitors (SMKIs) and additional 16 SMKIs which are approved by other government authorities. In oncology, 110 novel kinases as a target are explored, for which 45 targets of approved kinase inhibitors are developed so far [38]. Small molecule inhibitors are discovered, designed and synthesized by researchers to target ACK1. Various methods as fragment-based drug design, high-throughput screening, repurposing, and skeleton transitions are used for this purpose. Many inhibitors exhibited favorable pharmacokinetic activities and good anticancer activity, which can be used for clinical treatment of cancers. These drugs can be divided as (a) Selective Inhibitors, (b) Multikinase Inhibitors, and (c) Combination Drugs.

The chemical structures of few selective drugs are given in **Figure 4**. Compound 1 having IC_{50} (24 nM), is used to suppress pan cancer cells [39] through PTEN/AKT/mTOR signaling pathways [40]. Compound 2 and 3 has hindrance activity for ACK1. It was observed that the ACK1 inhibitory ability was not higher in Compound 4. Compound 5 is also a suitable drug with good pharmacokinetic properties. Compound 6 is a fragment based drug design with low water solubility. Though Compound 7, 8, 9, 10 have low pharmacokinetic activities, they can be used to provide reference to develop novel inhibitors for mutations in ACK1 tumors. Many other studied inhibitors are Pyrrolo [2,3-d]pyrimidine, Pyrazolopyrimidine, Imidazopyrazine and their derivatives [6].

We have used *in silico* approaches to study the pharmacokinetic properties of 14 multikinase inhibitors and its interaction to activated Cdc42-associated Kinase 1 (ACK1/TNK2) [41]. Many of these multikinase inhibitors are FDA approved therapeutic drugs targeting multiple targets for disease treatments. These drugs included the third generation dasatinib (5) [42], and bosutinib (6) [43] as an Abelson leukemia virus (ABL) and proto-oncogene tyrosine protein kinase Src kinase inhibitor. ADZ9291 (10) [44], Sunitinib (11), flavopiridol (12), gefitinib (13) [42] and compound 14 [45] has inhibitory effects on ACK1 (**Figure 5**).

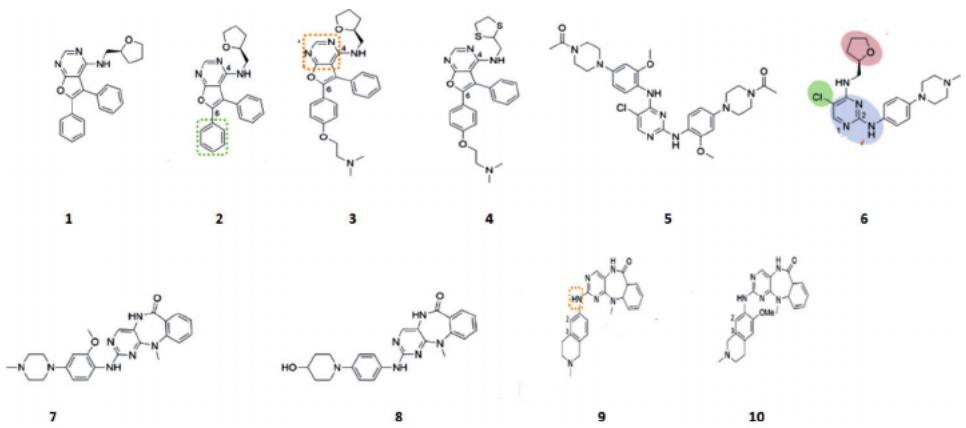

Figure 4.
Chemical structures of few selective inhibitors. The name of these selective inhibitors are AIM-100 (1), (2), (3), (4), KRCA-0008 (5), (R)-9b (6), XMD8-87 (7), XMD16-5 (8), benzopyrimidodiaze-pinone derivatives ((9), (10)). Adapted from Aoxue Wang et al. [6].

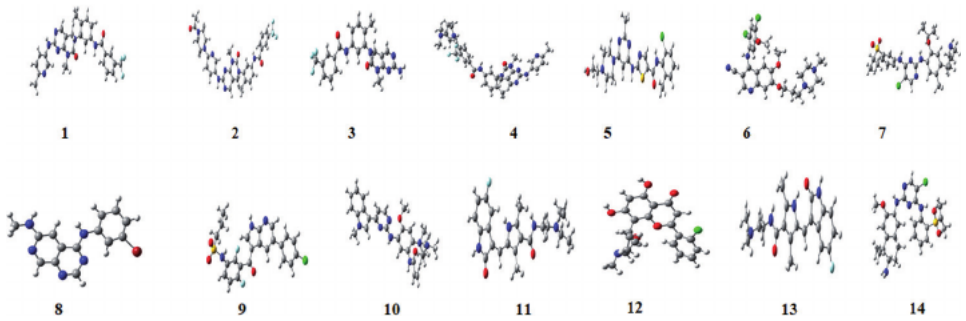

Figure 5.
Optimized structures of 14 multikinase inhibitors. The name for few multikinase inhibitors are GNF-1(1), Dasatinib (5), bosutinib (6), ceritinib (7), PD158780 (8), vemurafenib (9), ADZ9291 (10), sunitinib (11), flavopiridol (12), and gefitinib (13). Adapted from Srivastava [41].

As these drugs alone are not enough for the survival, so advances are made on the combination therapies for effective cancer treatment. The studied inhibitors showed better results when used in combination with other drugs.

2. Challenges

Though ACK1 is a therapeutic target for cancers, inflammation, immune and neurological diseases, very few inhibitors have entered the clinical trials. Hence there is urgent need to develop potential inhibitors. The *in vivo* pharmacokinetic properties of inhibitors also need to be improved. Some inhibitors have limited solubility in water which restricts the studies to be carried out on the animal models only. Due to the large distribution and participation of ACK1 in regulation of many signaling pathways, high specificity and precise positioning of inhibitors to diseased tissues are required, which increases the difficulty in drug designing. So, it is necessary to explore more biological functions of ACK1 and to verify the effectiveness of drugs *in vivo* and *in vitro*. As the inhibitors are developed by only limited methods (screening small molecules and fragment libraries), it have weak affinities which makes the selection of drug candidates difficult and time consuming. Further the development of allosteric inhibitors of ACK1 is also difficult as

it need full-length proteins in the biochemical analysis of ACK1, which is a great challenge as these proteins may exhibit aggregation, conformational changes and other phenomena, which are not possible for the *in vivo* and *in vitro* studies. In last 10 years, innovative immunochemotherapies have shown promising results in disease control rates but not survival. So, there is an acute need to develop novel drugs that can target dysregulated pathways in malignant tumors. Several functional challenges include the description of genetic abnormalities in the cancer kinomes and the recognition of accurate drivers which are accountable for tumor development. Only the precise analysis of the therapeutic involvement will indicate the clear role of kinases; as a tumor suppressor in non-cancer cells or a tumor mediator in cancer cells.

3. Application of inhibitors in drug repurposing

In oncology, repurposing of drugs means the reuse of already existing drugs to treat cancer rather than testing new drugs for the existing symptoms with malignancies. Introducing new drugs is a very time-consuming and costly process which requires many pre-clinical trials before its use for the commercial purposes. The existing drugs have a huge potential with untapped agents, which are clinically more relevant for disease treatment. More than 200 existing used off patent drugs have shown some evidence for anti-cancer treatment. Since these FDA approved drugs are not in larger number, it is better to repurpose the existing drugs for therapeutic purposes. These drugs can be repurposed for not only cancer treatment but also in rheumatoid arthritis and other disorders. Interestingly multikinase inhibitors are used to interact simultaneously many targets, these drugs can play an important role in drug repurposing for treatment of different diseases.

4. Future perspectives

Now it is well established that ACK1 is a promising target for tumor therapy and the clinical studies show that there is a strong correlation between the expression level of activated ACK1 and prognosis and progression of cancers. Six specific inhibitors with high affinity for ACK1 has been identified which showed potential inhibitory activity. Some inhibitors also showed good pharmacokinetics properties *in vivo*. It has been observed that light-controlled PROTACs degrade specific proteins at certain locations in the body, so novel ACK1 inhibitors could have a local impact on pathologic tissues by light control. Fortunately, immunotherapy has been considered as an alternative tool for cancer patients. The treatment included many checkpoint inhibitors as nivolumab, pembrolizumab, and atezolizumab. Many other inhibitors as dasatinib, nilotinib, bosutinib along with imatinib mesylate has also used as chemotherapeutic agent for treatment in chronic myeloid leukemia (CML) patients. Considering these problems, Allosteric inhibitors, inhibitors targeting different structural domains of ACK1, inhibitors having blocking interactions within proteins, Proteolysis targeting chimeras (PROTACs), Combination therapies and dual-target drug complexes need to be develop in future. Moreover, many ACK1 interacted proteins or substrates need to be identified which can be utilized for precision medicine in cancer patients. The implementation of bioinformatics based methodologies as structure based drug designing can definitely help in drug delivery precision medicine for cancers. Refinement of effective compound screening and profiling technologies, and natural compounds need to be explored to reduce the off-target toxicity. Allosteric and covalent inhibitors, and targeted

degraders such as PROTACs and molecular glues will be the next players of kinase drug discovery in future.

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Gene Signature-Based Drug Repositioning

Zhilong Jia, Xinyu Song, Jinlong Shi, Weidong Wang and Kunlun He

Abstract

With the advent of dynamical omics technology, especially the transcriptome and proteome, a huge amount of data related to various diseases and approved drugs are available under multi global projects or researches with their interests. These omics data and new machine learning technology largely promote the translation of drug research into clinical trials. We will cover the following topics in this Chapter. 1) An introduction to the basic discipline of gene signature-based drug repurposing; 2) databases of genes, drugs and diseases; 3) gene signature databases of the approved drugs; 4) gene signature databases of various diseases; 5) gene signature-based methods and tools for drug repositioning; 6) new omics technology for drug repositioning; 7) drug repositioning examples with reproducible code. And finally, discuss the future trends and conclude.

Keywords: transcriptome, databases, drug repurposing, mode of action, reproducible study

1. Introduction

Drug repositioning is to identify new indications of the approved drugs. It has lower risk, less human resources, lower cost, and shorter developmental period, compared with traditional drug development. Sir James Black, a Nobel Prize laureate, originally stated that "The most fruitful basis for the discovery of a new drug is to start with an old drug", largely promoting the concept of drug repositioning [1]. There are huge examples of drug repositioning as described in the book. Multinational pharmaceutical companies, such as AstraZeneca and GSK, also showed their great interest in drug repurposing approaches [2, 3].

In this Chapter, we focus on gene signature-based drug repositioning. The idea could date from 2000 year. Hughes et al. built a prototypical library of the microarray-based gene expression signatures of Yeast with about 300 diverse gene mutations and the treatment of 13 drugs with known molecular targets by keeping other experimental conditions consistent [4]. They identified a new target of the drug dyclonine by comparing the signatures of genes and drugs via pattern matching [4]. This article opened a door for gene signature-based drug repositioning [5].

A comprehensive gene signature library of genes, diseases and perturbations plays a fundamental role in gene-signature-based drug repositioning. From the genes' view, the knocking down, knocking out, knocking in genes could be achieved to represent the expression signatures of genes with the advances of molecular biology, especially the emergence of the RNAi and CRISPR/Cas9 technology [6].

From the diseases' view, modeling disease in a cell or animal experimental assay would make it possible to produce the gene signatures of various diseases via the quantification of molecular phenotypes. It should be noted that modeling various diseases in parallel and high throughput ways are relatively difficult so far as the condition of modeling various diseases is disease-specific or unclear due to the complexity and our little understanding of some diseases. However, with the development of the pathogenesis of various diseases, it will be efficient to model cellular and animal models of various diseases by magic genome editing using CRISPR/Cas9 technology [7].

Finally, from a drugs' view, there are thousands of approved drugs available so far. Lots of the bioactive compounds, besides the approved drugs, were also tested to obtain their gene signatures. Particularly, the connectivity map (CMap) [8] and Library of Integrated Network-based Cellular Signatures (LINCS) program [9, 10] largely promoted the rapid development of drug repositioning as they provided a huge of gene signatures of drugs and compounds freely available to the scientific community.

The core principle of gene signature-based drug repositioning is that the candidate drugs should revert the gene signature of the disease of interest, which is changed by the disease, compared with the controls (**Figure 1**). The reversion could be characterized by anti-correlation, distance, similarity and metrics produced by machine learning models. A derivative principle is that the similarity of two drugs could reveal similar indications of the two drugs. In detail, if drug A could be used to treat disease C, and the other drug B is similar to drug A based on their gene signatures, then drug B could also be used to treat disease C. This idea should come from chemoinformatics as the principle that similar drugs based on chemical structures should have similar functions is widely used in the field of drug research and development, especially the development of me-too drugs [11]. Importantly,

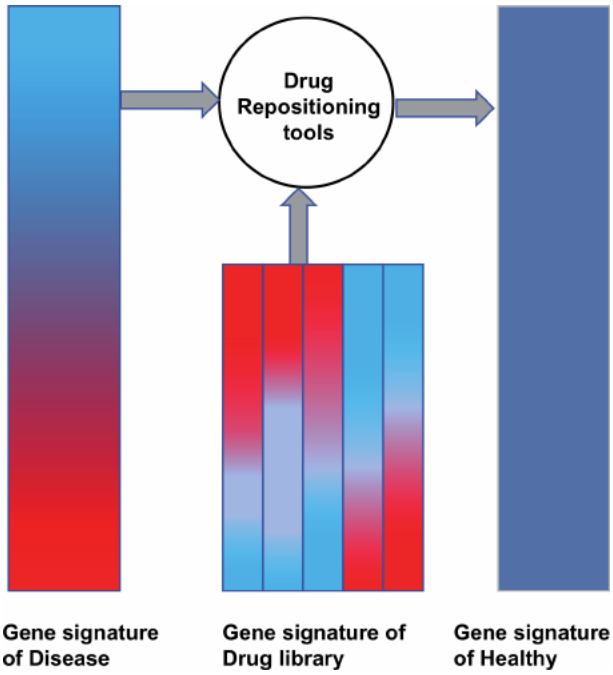

Figure 1.
The core idea of gene signature-based drug repositioning. Drug repositioning tools search the gene signatures of a drug library to identify which signature is "opposite" to the gene signature of disease, reverting the state of disease to the healthy state.

several researchers have developed or detailed this principle from different perspectives, making this idea efficient to implement and use.

The gene signatures are the molecular phenotype, revealing the molecular landscape of genes, diseases or drugs. In general, the gene signatures are the expression profiles or changes of RNA measured by RNASeq-based transcriptome via microarray, Next-Generation Sequencing or Third-Generation Sequencing [5, 8]. More broadly, the gene signature could be the abundance profiles or changes of proteins qualified by the antibody-based or tandem mass spectrometry (MS/MS)-based proteome. The reason why is that the principle of gene signature-based drug repositioning is suitable to any molecular phenotype, such as the transcriptome and proteome. Moreover, in machine learning models, the tabular data of transcriptome and proteome is similar to a great extent as they are features of samples in a high-level and united view.

In summary, with the rapid advance of various omic technology, a huge amount of public available omic data related to molecules, drugs, diseases and genes, computational resources and efficient deep learning algorithms make the field of drug repositioning vigorous. There will be increasing therapeutic applications of drug repositioning. In the following sections, we will introduce the databases related to genes, pathways, drugs and diseases, providing the resources for gene signature-based drug repositioning, then describe key tools for web servers for drug repositioning with a highlight on the new powerful and easy-to-use methods, show examples for drug repositioning for several diseases with reproducible code, convenient to the readers to follow. Finally, we will summarize the ongoing challenges, unmet needs, future trends and conclude.

2. Databases of genes, pathways and drugs for drug repositioning

Genes play a critical role in gene signature-based drug repositioning. Especially, the targets of drugs are of importance in traditional drug development. In General, the targets of drugs are human or viral proteins, which are druggable [12] and associated with a particular disease or multi diseases. So far, there are about 900 biomolecules targeted by about 1500 US FDA-approved drugs as curated by Rita et al. [13]. Obtaining this information will facilitate the process of gene signature-based drug repositioning. Some databases and web servers have gene information, which are useful in drug development [14].

GeneCards (https://www.genecards.org/) is an integrative knowledge base and web server with comprehensive information on all human genes, scratching more than 150 high-quality web sources, from genotype to phenotypes and functional information [15]. Though it is a general database, which is not centric on drug development, it provides comprehensive knowledge about a gene of interest. It is highly recommended to browse this website at the beginning of a study of a target.

DGIdb (drug-gene interaction database, www.dgidb.org) is a webserver with drug-gene interaction and druggable genes information, collected from more than thirty high-quality web sources [16]. If biomarkers or therapeutic targets are identified, then researchers could search which drugs could target the biomarker or therapeutic target using DGIdb, achieving a quick translational opportunity.

The Open Targets database (https://www.opentargets.org/) aims to identify and prioritize promising therapeutic targets of drugs by analyzing human genetics, genomics and functional genomics data [17, 18]. The database emphasizes the importance of genetics of diseases via genome-wide association studies to approach gene causal inference, which is beneficial to drug development [19, 20].

The Clue.io webserver (https://clue.io/) includes the updated CMap LINCS gene expression resource perturbed by CRISPR gene over-expression, RNAi gene knock-down and CRISPR gene knockout generating loss-of-function mutants [9, 21]. This webserver has abundant data about the gene perturbation, providing a great resource to study the effect of a target, mimicking the targets affected by drugs [22–24]. Meanwhile, it also supplies a drug repositioning hub for researchers, a curated library of drugs with a companion knowledge resource [25].

Pathways, besides gene level, could also be a key resource in drug repositioning. Pathway, consisting of a set of genes, could be the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, gene ontology (GO), Reactome Pathway Database (https://reactome.org/) and other gene sets. As genes in a pathway are not randomly selected, a generalized pathway concept is the gene set, substantially enlarging the function aspects of pathways. A good resource of the gene sets is the Molecular Signatures Database (MSigDB, https://www.gsea-msigdb.org/gsea/msigdb/) as it supplied a downloadable gmt-formatted gene set dataset, facilitating its use in the bioinformatic analysis [26]. Several reasons highlight the importance of the pathway. Firstly, it could be used to illuminate the mode of action of drugs by connecting the genes and drugs [27]. Secondly, it could be a feature summarizing the gene-signature at a higher level, which is useful in machine learning-based modeling. It is different from the gene level as it captures different information about drugs or diseases [28–30]. Thirdly, the pathway analysis could enhance the confidence of the prediction of the candidate drugs [31].

The information about drugs is an invaluable resource to drug repositioning and an evaluation dataset of drug repositioning. The repoDB database is a standard dataset to benchmark various computational repositioning methods, which consist of 6677 approved and 4123 failed drug-indication pairs [32]. The Experimental Knowledge-Based Drug Repositioning Database (EK-DRD, http://www.idruglab.com/drd/index.php) curated 1861 FDA-approved and 102 withdrawn drugs with validated drug repositioning annotations [33]. These datasets will facilitate the training and testing of the machine-learning-based models.

3. Gene signature databases related to drugs

The gene signature databases of drugs and compounds are fundamental resources determining the searching space for drug repositioning. For a long time, researchers have been pursuing the enlargement of the gene signature library of drugs and compounds. For example, researchers have explored a bunch of bioactive compounds and ligands, such as growth factors and cytokines, which are not drugs but with known functions [8–10]. There are lots of data resources related to drugs. The sources of these data are mainly from two aspects. One is the public data, such as GEO, which is scattered in the database. A manual curation by professional researchers is necessary to make a usable dataset for drug repositioning. There is a trend for advanced metadata curation from the GEO [34]. The other one is from large projects, such as CMap, aiming to create a reference dataset of gene signatures for drug development.

NCBI GEO [35], EMBL-EBI ArrayExpress [36] and NGDC Gene Expression Nebulas [37] store massive omics data, including many transcriptome data of drugs and other compounds. But researchers need to search, collect and tidy them before their use for drug repositioning. Fortunately, several groups have collected multi-gene expression signatures related to the drugs.

The CREEDS (CRowd Extracted Expression of Differential Signatures) extracted and analyzed the signatures of 875 drugs and 828 diseases from GEO

via a crowdsourcing project, setting in a massive open online course on Coursera [38]. The dataset could be downloaded from the website, https://maayanlab.cloud/CREEDS/.

HERB (http://herb.ac.cn) is a high-throughput experiment database of traditional Chinese medicine, consisting of 7263 herbs and 49,258 ingredients, from 472 high-throughput GEO datasets, providing complementary and valuable drug resources [39].

The CMap version 1 (https://portals.broadinstitute.org/cmap/) consists of Affymetrix-based 6100 gene signatures of 1309 compounds perturbing five different cell lines (such as PC3, MCF7, HL60) with varying doses (mainly 10 μM). Notably, there were 164 distinct perturbagens, including approved drugs and non-drug bioactive compounds, in the original article published in the *Science* journal [8]. Indeed, this dataset stimulates the rapid development of drug repositioning, indicated by the high citations (more than 1800 times). It suggests the great value and success of a large-scale community Connectivity Map project.

The CMap version 2 (https://clue.io/cmap), belonging to NIH's Library of Integrated Network-Based Cellular Signatures (LINCS) program, includes 1.3 million L1000 profiles and 25,200 unique perturbations on variable cell lines [9]. They used L1000 technology due to the cost and argued that about 1000 landmark genes could recover 82% of the information in the full transcriptome based on a comprehensive comparison [9]. As expected, the updated dataset also motivated the continual development of drug repositioning. It should be noted that the consistency between the two versions of CMap is not high with a low recall [40]. It suggests that drug repositioning based on the CMap should consider other evidence to filter false positives in the computational drug repositioning.

In summary, the availability of huge gene signatures of drugs makes the gene signature-based drug repositioning possible as a big data basis. Meanwhile, researchers are still developing new transcriptome technology to make the large-scale transcriptome sequencing of millions of drugs treating different cell lines with various doses possible at a relatively low cost. In addition, with the cost of conventional RNASeq lower, it is also possible to use the RNASeq directly soon.

4. Gene signature databases of various diseases

The gene signature databases of various diseases are a complementary resource to drug repositioning. Importantly, the gene signatures of diseases are robust across different tissues and experiments to some extent (Dudley et al. 2009). As mentioned in the introduction section, it is difficult to apply a high-throughput way to model various diseases in parallel. Researchers have collected some gene signature datasets related to numerous diseases. However, in practice, biologists usually focus on a specific disease, which means that they could obtain the gene signature of the disease by themselves. Once they have the gene signature of the disease, they could directly query the gene signature library of drugs to get the candidate drugs for this disease.

The gene signatures of diseases were mainly collected from the GEO. ADEPTUS (Annotated Disease Expression Profiles Transformed into a Unified Suite) supplied about 14,000 ready-to-use gene signature profiles, annotated with Disease Ontology terms [41]. ADEPTUS built a classic way to form a gene signature of various diseases. The STARGEO (Search Tag Analyze Resource for GEO) project generated annotations of disease-related samples in GEO to identify robust signatures of disease by meta-analysis via a crowdsourcing approach [42]. It covered about 250 types of diseases and could be improved via the webserver. The DrugVsDiseasedata

(Drug versus Disease data) package defined 45 gene signatures of diseases, such as Breast with Small-cell Lung, Cervical, Bladder and Prostate cancer, collected from GEO [43]. Recently, Porcu et al. reported that differentially expressed genes reflect disease-induced rather than disease-causing changes in the transcriptome via the Mendelian randomization method. Thus, identifying the upstream genes, which cause the diseases, would be a promising direction in the transcriptome data of diseases.

Although, there are several gene signature datasets of diseases, more efforts are necessary to enlarge the library of the types of diseases. The disease ontology is a fruitful resource for reference when searching for a disease. With the scale of gene signatures of diseases increasing, there will be more possibility of connecting drugs and diseases as the searching space for the algorithm is expanded.

5. Gene signature-based methods and tools for drug repositioning

Once the gene signatures of drugs and diseases, as well as other useful information (such as the structure of drugs), are ready, we could make a computational drug repositioning analysis. In the end, it is to find a method to connect the drug and disease. This connecting method could be a similarity metric [44], community discovery, matrix factorization and completion, machine learning-based models and so on. A good method should significantly enrich true positive results and deplete false-positive results.

There are several biologist-friendly web servers, convenient to use without the need for programming. The CMap version 1 website is one of the most popular websites in the field of drug repositioning. The CMap version 2 website supplies a more fruitful website. The enrichr website (https://maayanlab.cloud/Enrichr/) also provides the drug repositioning module with the drug and disease libraries (for example, Drug_Perturbations_from_GEO_down gene set) [45, 46]. Biologists could easily use these websites for drug repositioning without programming.

The nonparametric Kolmogorov–Smirnov statistic, formalized in Gene Set Enrichment Analysis (GSEA), was used in the original CMap article, indicating its power [8, 47]. It tests whether the empirical distribution of data (a set of genes) is different from a reference distribution (such as a ranked gene list related to a drug). The nonparametric test simplifies the statistical test process, making it feasible to multi situations.

PAGE (parametric analysis of gene set enrichment) was more sensitive and less-computational than GSEA [48], which could be used to evaluate the similarity between two gene expression signatures. Dr. Insight used the concordantly expressed genes in a frame-breaking statistical model to connect the drug and disease [49]. The eXtreme Sum (XSum) was a similarity scoring algorithm, which was developed by Jie et al. It showed a better performance than the KS statistic based on the area under the curve using 890 drug-indication pairs with 496 compounds and 238 disease signatures [50].

Network-based community discovery could exploit the similarity in gene expression signatures of drugs and identify the similar drugs, which should be clustered together [51]. They also implemented a tool, MANTRA (Mode of Action by NeTwoRk Analysis), which was accessible and biologist-friendly at http://mantra.tigem.it [52]. GPSnet (Genome-wide Positioning Systems network) associated the drug and the gene signature-based disease modules in the protein–protein interactome network [53]. DeMAND (detecting mechanism of action by network dysregulation) developed a regulatory network-based approach to elucidate the MoA using gene expression signatures [54]. Chemical Checker integrated five-level

data of drugs, such as targets, morphology and gene expression signatures, to evaluate the similarity of the drugs via the dimensionality reduction and network embedding algorithm [55].

Cogena, co-expressed gene-set enrichment analysis, focused on the idea of targeting co-expressed genes instead of all the differentially expressed genes for drug repositioning [27]. It empowered simultaneous, gene set knowledgebase-driven drug repositioning analysis and illustrated the mode of action of the predicted drug and disease pairs. Cogena has been widely used in drug repositioning for several diseases, including psoriasis, Coronavirus Disease 2019 (COVID-19) [56, 57], Crohn's disease [58], periodontitis [59].

Machine learning, especially deep learning algorithms, are suitable to the gene expression signatures inherently. The low-rank matrix approximation and randomized algorithms were used in drug repositioning by filling out the unknown connection in the drug-disease pairs [60] The iDrug could reposition drugs via a cross-network embedding and transferring knowledge from the drug target information [61]. DLEPS (deep learning-based efficacy prediction system) used one-dimensional convolutional neural networks to learn the relationship between the structure of drugs and gene expression signatures to predict drug efficacy [62]. Clearly, with the advances of deep learning, especially the graph neural network, lots of innovative algorithms will be continually applied in the drug repositioning field to improve performance.

6. New high-throughput technology for drug repositioning

Researchers try to develop new high-throughput RNASeq technology to improve the precision of transcriptome with the constraint of cost. For example, the microarray was used in the first version of CMap, while the L1000 technology was used in the second version of CMap, that is LINCS with a more than 1000-fold scale-up of the CMap. Via a Luminex bead-based probe hybridization, the L1000 only measured the mRNA abundance of 978 "landmark" genes with the expression of the remaining gene inferred by a machine learning algorithm [9]. This selection largely resulted from lowering the cost of obtaining the transcriptome of a huge scale of drugs and compounds.

RNA-Seq via Next-Generation Sequencing is a relatively new emerging technology in the drug repositioning field. Due to the higher cost, researchers tried to maintain the transcriptome performance when lowering the cost in several ways. For example, a subset of genes with a reduced representation of the transcriptome could be sequenced instead of all the mRNA. The L1000 technology used the most informative genes, named "landmark" genes [9]. Deepak et al. argued that a knowledge-driven subset of 1500 sentinel genes could precisely predict pathway perturbations [63]. RASL-seq (RNA-mediated oligonucleotide annealing, selection, and ligation) only measured hundreds of pre-defined genes in response to a set of 350 chemicals and their mixtures, which provided a cost-effective approach to quantify gene expression signature with a panel of marker genes [64]. TempO-Seq, Templated Oligo assay with Sequencing readout, could determine the whole transcriptome via a targeted way, requiring less sequencing depth [65].

The pooled and low-depth Next-Generation Sequencing is another approach to lower the cost but maintain the performance. PLATE-seq (pooled library amplification for transcriptome expression) introduced the sample-specific barcodes, allowing pooled library construction in 96 wells and low-depth sequencing, which is about 15-fold less expensive than canonical RNA-Seq [66]. DRUG-seq efficiently captured transcriptional changes with low-depth reads by importing cell barcode

and Unique Molecular Index (UMI) in 384- and 1536-well format with fewer steps, compared with PLATE-seq [67]. Notably, DRUG-seq also supplied an open-source R program analysis pipeline at Github recently [68]. BRB-seq (Bulk RNA Barcoding and sequencing) used early-stage multiplexing to produce 3′ cDNA libraries for multi-samples, while with a lower cost [69]. 3'Pool-seq was an optimized cost-efficient method of transcriptome profiling, which was also adapted for a 96-well plate format and ERCC spike-ins. Collectively, researchers have developed multi new transcriptome technologies while lowering the cost of sequencing to implement the RNASeq for large-scale samples, which could be due to the different doses, different treatments, and different periods of treatment.

Other types of gene signatures, such as the proteome and metabolome, could also be used in drug repositioning. Zhao et al. created a systematic map of protein-drug connectivity that compiled 210 clinically relevant protein signatures based on antibody-based proteomics technology in more than 12,000 cell-line samples in response to about 150 drugs [70]. ProTargetMiner was a proteome signature library of 56 molecules in A549 cancer cell lines, forming a valuable tool in drug discovery [71]. Benjamin et al. profiled the proteomes of five lung cancer cell lines (such as A549, Calu6 and Calu1) perturbed by more than 50 drugs based on the label-free proteomics platform [72]. Moreover, an atlas (http://bbmri.researchlumc.nl/atlas/) of 87 drugs and 150 clinically relevant plasma-based metabolite associations will contribute to the drug development as well [73]. Other omics data, besides transcriptome, related to drugs and diseases will promote the drug repositioning flourishing. In summary, new omics technology will precisely quantify the signatures related to drugs and diseases with a low cost, permitting the large-scale omics project, enlarging the searching library for drug repositioning.

7. Drug repositioning examples with reproducible code

Due to the pandemic of COVID-19 and no effective drugs for this disease, drug repositioning is a great way to combat this disease. Several researchers have used cogena for drug repositioning to fight the COVID-19 [56, 74].

We used the metatranscriptome data of the bronchoalveolar lavage fluid from 8 severe COVID-19 patients and 20 healthy controls to obtain the gene expression signature of COVID-19 [75]. The co-expression analysis, pathway analysis and drug repositioning analysis were done using the cogena pipeline [56]. We identified several drugs which were associated with COVID-19 reported before. For example, Saquinavir, a protease inhibitor, is a drug for human immunodeficiency virus infection. This drug was also identified by several docking methods [76]. Dexamethasone is a "major development" in the fight against COVID-19 in the RECOVERY trial [77]. Ribavirin can be used to treat SARS-CoV and MERS-CoV infections [78]. Importantly, it is a recommended drug in the diagnosis and treatment protocol for COVID pneumonia (trial version 5–latest) published by the National Health Commission of the P.R. of China. It was also identified by several docking methods [79]. Furthermore, we identified several other candidate drugs for COVID-19, for example, dinoprost, a smooth muscle activator, and (−)-isoprenaline, a bronchodilator for obstructive lung diseases. These candidate drugs could be tested in vitro and in vivo to validate their possibility.

The whole pipeline of this gene-signature-based drug repositioning for COVID-19 using cogena is accessible at https://github.com/zhilongjia/COVID-19 with data and code, forming a good resource for drug repositioning and reproducible study.

There are also other examples of drug repositioning using cogena with reproducible codes. For instance, the code of the drug repositioning for psoriasis is

available at https://github.com/zhilongjia/psoriasis and the code of drug repositioning for periodontitis is available at https://github.com/zhilongjia/Fn_HGFcell. These examples will enhance our understanding of how drug repositioning works and how to implement drug repositioning.

8. Future perspectives and conclusion

The future of gene signature-based drug repositioning is bright. The booming biotechnology and pharmaceutical industry, especially the emerging sequencing and MS field, supplies an important motivation to sequence more omics data related to drugs and diseases. The artificial intelligence industry, particularly the deep learning algorithm, will also promote the rapid development of the drug repositioning field as it will improve the rate of the true positives and lower the rate of false positives. The omics data of drugs and diseases is like electricity, while the algorithm is like a machine. The seamless combinations of them will produce new opportunities for gene signature-based drug repositioning. More data means a larger searchable space to identify the new relationship between drugs and diseases. Additionally, the signatures-based combination of drugs could also be investigated to deal with intractable diseases. Meanwhile, more evidence from different aspects of the drug-disease pairs will improve the quality of perdition.

In the end, we highlight the key points of this chapter.

1. A systematic introduction to gene signature-based drug repositioning and the core principle of gene signature-based drug repositioning;

2. Gene signature could be achieved based on molecular phenotypes, such as transcriptome and proteome;

3. Basic databases of gene, pathway and drug for drug repositioning;

4. Gene signature databases of drugs and diseases

5. Gene signature-based methods and tools for drug repositioning;

6. New high-throughput technology for drug repositioning;

7. Drug repositioning examples with reproducible code;

8. The future direction of gene signature-based drug repositioning.

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Conflict of interest

The authors declare no conflict of interest.

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Recent Progress in Drug Repurposing Using Protein Variants and Amino Acids in Disease Phenotypes/Disorders

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Abstract

Life is constituted of large group of macromolecule, functional and structural called "Protein," made of amino acids (AA), and linked with peptide bonds with specific protein unique sequences. Variations in proteins are thought to have diverse effects with consequences on structure, stability, interactions, pH, enzymatic activity, abundance and other properties. Variants can be of genetic origin or it could occur *de novo* at the post-translational protein level. The sequence of amino acids defines protein structure and functions. Protein is involved in several critical functions like the physical cell-cell communication. Breakthrough in molecular science has shown that, to develop drugs for managing a disease-associated variations requires understanding of consequences of variants on the function of the affected protein and the impact on the pathways, in which protein is involved. Using biophysical/bioinformatics methods, immense amount of variation data generated is handled-connected to disease phenotypes. Obviously, there remain continuous needs for the combinations of genetic probing methods/bioinformatics, to predict single-nucleotide variations (SNV), for effective rational drug design that would embrace naturally occurring bioactive components of plant origin, towards the effective management of disease phenotype emanating from protein and amino acid variations. This, well thought out and synchronized concept, remains a way forward.

Keywords: protein variation, epigenetics, disease management, single nucleotide variation, protein variants, amino acids in disease phenotypes/disorders

1. Introduction

Variations in the genome and protein expression remain a driver of most diseases with their polygenic phenotypes. Diseases may manifest with time emanating from aberrant protein expression. These biochemical processes are complex in nature, as it involves molecular interactions at both the DNA-RNA/protein level. Within the context of protein-protein interaction (PPI), it has become essential to look at the long-term clinical goal, which could be to identify disease-specific patterns of PPIs, which could serve as a disease- or treatment-responsive biomarkers whose selective measurement may lead to improved diagnosis or prognosis for common human disorders [1].

Interests in the study of variations in DNA/protein have helped to espouse factors germane to influencing biological processes and genome stability. This is more so bearing; genomic integrity is particularly important as they provide the blueprint for the next generation [2]. During cell division, homeostasis is required, and for human health, the genome needs to be copied prudently such that a copy of each chromosome is passed on to the daughter cells. The polygenic nature of some disease phenotypes, controlled by a combination of several genes all playing together makes it essential to unravel biological processes in a piecemeal. For instance, most diseases with a number of persons with inherited predispositions including, heart disease, arteriosclerosis, and some cancers are thought to be polygenic [3].

Looking at these processes within the context of ribonucleic acid (RNA) on the other hand presents different facts. At some point in time, RNA was thought to be much less important than DNA since it did not carry any of the genetic characteristics of an organism. However, lately, it has become obvious that this might not be entirely so bearing, the life cycle of RNA viruses for instance is directed to transport, multiply, and deliver the viral RNA genome into other cells. Fortunately, not all of these viral genomes can encode all proteins in the cell that are required for these known processes to be accomplished. Thus, overcoming this limitation, viruses are known to hijack cellular RNA-binding proteins (RBPs) [4, 5].

Responding to such invasion, host cells do concertedly employ specialized RBPs as a detection mechanism for viral RNAs and their intermediates of replication through the recognition of the molecular signage such as the under-methylated, cap triphosphate ends, and double-stranded RNA (dsRNA) [6]. Beyond this, several other observations have been made [3, 4], highlighting the essential role that RBPs play in regulating the viral life cycle. For instance, it is thought that RBP sensing of viral RNA triggers the cellular antiviral state, which can suppress viral gene expression [6], leading to the inhibition of protein synthesis and the production of interferons [4, 5].

Recently, using multiple proteome-wide approaches [7] had identified RBPs involved in the SARS-CoV-2 life cycle whilst showing that the repertoire of cellular RBPs widely remodels in response to SARS-CoV-2 infection, via proteins involved in antiviral defenses, RNA metabolism, and other pathways.

In all of these processes, transcription factor (TF) mutations have been studied for decades, with RBPs being overlooked as drivers of disease and as therapeutically relevant targets. Now it is established that RBPs determine the fate of transcribed RNAs by regulating their splicing, polyadenylation, translation, subcellular localization, and turnover [8].

For drug repurposing, diseases that are driven by a known or combination of mutants at the protein level are of major attention for direct targeting. Moreover, changes in cellular growth rate and the identity that occur during diseases such as cancer, hemoglobinopathy, etc., are, known to be driven by specific gene expression signatures that are programmed by the activity of DNA-binding TFs and RBP [9]. From recent findings, it is now clear that RNA-binding proteins (RBPs) are critical regulators of post-transcriptional gene expression [9]. Within this context, Liu and Shi [10] earlier established the importance of RBP in Amyotrophic lateral sclerosis (ALS), disease progression. Establishing that the heterogeneous ribonucleoproteins (horn A2/B1) mutation in patients with ALS did not just disable the protein, but instead, the mutation conferred some new toxic properties that scrambled RNA processing, fast-tracking the death of motor neurons [10].

Some other known fact is that missense mutation is a mistake in the DNA and it could arise due to aberrant TF. Missense mutations for instance in tumor suppressors result in its loss of function (LOF) in a variety of manners including loss of stability of the protein or the disruption of a crucial ligand/DNA/protein binding site [11]. The Worldwide Protein Data Bank (wwPDB) have over 88,000 protein

structures, many of which play vital roles in critical metabolic pathways that may be regarded as potential therapeutic targets and specific databases containing structures of binary complexes [12]. Moreover, a recent breakthrough in molecular science has shown that the key to developing targeted therapy, for disease-associated variations is with the critical understanding of the consequences of that variant on the function of the affected protein, and the impact on the pathways in which that protein is involved [9]. Proteins are produced and recycled by some critical processes in their tissue sources and are degraded into necessary amino acids through very controlled bio-signaling and feedback systems. For instance, the salvage pathways are known as a major source of nucleotides for the synthesis of DNA, RNA, and enzyme co-factors.

The disproportion of protein demand, dietary supply, and productions do result in a variety of disease phenotypes due mainly to deficiency, occasioned sometimes by variation properties. A critically important enzyme of purine salvage in rapidly dividing cells for instance is adenosine deaminase (ADA), which catalyzes the deamination of adenosine to inosine. Deficiency in ADA results in the disorder called severe combined immunodeficiency (SCID). This is a genetic disease amongst many others that is characterized by the development of nonfunctional T and B cells due to genetic mutation resulting in heterogeneous clinical phenotype [13].

2. DNA-protein interactions

The cis-regulatory DNA elements' interactions with the transcription factors seem to be critical components of transcriptional regulatory networks [14]. The genome with the complete cDNA sequences contains large numbers of transcription factors with their binding DNA sequences. It is expected that a comprehensive analysis of DNA-transcription factor interactions will provide a deep understanding of the mechanisms of drug metabolism in critical processes such as cell proliferation, developmental processes in tissue morphogenesis, and disease manifestation [14]. The combined use of chromatin immunoprecipitation (ChIP) assay with DNA microarrays (ChIP-chip) [14, 15] are the most widely used high-throughput method for discovering non-coding region but important (cis-regulatory) DNA elements for a transcription factor [16]. Albeit, the development of high-throughput methods for discovering transcription factors for DNA regulatory elements remains in its infancy, even though the yeast one-hybrid method [17] and phage display [16] are attractive candidates, in this regard. However, these methods have some shortcomings including, they are not easily scalable because of the use of living cells. Further, the overexpression of transcription factors are thoughts to affects cellular metabolism, and as such, making transcription factors difficult to screen. Thus, to avoid these difficulties, focus totally on in-vitro mRNA display technology such as in-vitro virus (IVV) method [11, 16] for the discovery of DNA-protein interactions serve as a good alternative.

To map out the transcriptional regulatory networks at a wider genome level, a comprehensive analysis of DNA-protein interactions is important Thus, the IVV method had been employed for in vitro selection of DNA-binding protein heterodimeric complexes [18]. Using improved selection conditions, enhanced with a TPA-responsive element (TRE) as a bait DNA, known interactors such as; c-fos and c-jun were simultaneously enriched about 100-fold from a model library (a 1:1:20000 mixture of c-fos, c-jun, and gst genes) after one round of selection [18]. Moreover, the AP-1 family genes, including c-jun, c-fos, junD, junB, atf2, and b-atf, were successfully selected from an IVV library constructed from a mouse

brain poly A+ RNA after six rounds of selection [19]. These results indicated that this method (IVV selection system) have the potential to identify a variety of DNA binding protein complexes in a single experiment. Since almost all transcription factors form hetero-oligomeric complexes towards binding with their target DNA, this method should be most useful to search for DNA-binding transcription factor complexes [11, 16], which will further illuminate the understanding of drug repurposing in disease state conditions.

3. Diseases arising from mutations

Numerous computational tools have been developed for the interpretation, analysis, and prioritization of variations and their effects [20]. Many DNA/protein variations and disease-causing mutation databases are now available for references. For instance, the locus specific variation database (LSVD) is present at Leiden Open Variation Database (LOVD) system for all human genes [21]. Although some of the databases seem to contain similar information, however, the LSDBs are listed at the Human Genome Variation Society (HGVS) Website (http://www.hgvs.org/locus-specific-mutation-databases), the LOVD site (http://grenada.lumc.nl/LSDB_list/lsdbs), the GEN2PHEN server (http://www.gen2phen.org/data/lsdbs) [22], and at the Web Analysis of the Variome (http://bioinformatics.ua.pt/WAVe/) [20, 23, 24].

Besides the above databases, there are many others that were most recently covered ([20, 25], and the references therein). Moreover, recent advances in genome-wide association studies, next-generation sequencing technologies coupled with genetic linkage analysis have enhanced output in the analysis of mutation-causing diseases. Many of these methods are useful for detecting single-nucleotide polymorphisms (SNPs), which are found to be common in aberrant gene functioning. However, it may also be noted, the majority of structural variations (SVs) that occur in the human genome are yet to be fully characterized by single short-read platforms [26]. Suffice, for many genetic diseases, association studies have relied most heavily upon short read, high throughput sequencing technologies [27, 28].

Some `genetic variations with the consequence encoded proteins are known to manifest into disease phenotypes with the deleterious outcome to the patient. Within these are hemoglobinopathy including sickle cell disease (SCD), which are caused by a single germ-line mutation substituting (A to T) in the codon for amino acid 6. The change converts a glutamic acid codon (GAG) to a valine codon (GTG) [29, 30].

3.1 Single mutation as a lead cause of amyotrophic lateral sclerosis (ALS)

Most recently, due to the advances mentioned above, it led to the finding that a mutation in the C9orf72 gene (chromosome 9 open reading frame 72 genes) is the primary genetic cause of amyotrophic lateral sclerosis (ALS). These losses of function, induced by the mutation of the C9orf72 gene are thought to affect communication between motor neurons and muscles in people with ALS [31]. Further, this mutation is thought in part to be responsible for 40–50% of hereditary cases of ALS, and 5–10% of cases without family history. This mutation consists of an expansion of a sequence of hexanucleotide (GGGGCC) DNA bases, going from a few copies (less than 20 in a healthy person) to more than 1000 copies [30]. Until now, it still remains unclear how this GGGGCC base repeat expansions cause neurodegeneration in ALS. Although, mechanistically, the C9orf72 protein function in a complex with the WDR41 and SMCR proteins (guanine exchange factors (GEF)) for Rab8 and Rab39 [31].

In a more recent study, the gene C9orf72 role on the protein TDP-43 (transactive response DNA binding protein-43) was revealed. The TDP-43 protein plays an important role in ALS. It is thought that the C9orf72 gene may affect the protein TDP-43's location within the cell. "In approximately 97% of ALS patients, it is being observed that the TDP-43 protein is depleted from the nucleus, forming aggregates in the cytoplasm rather than being in the nucleus, as is the case in healthy people [26, 32, 33].

The average incidence rate of ALS worldwide is about one in 50,000 people per year and the average age of onset of the disease is about 60 years, with men at a slightly higher risk compared to women. FDA-approved treatments for ALS are only modestly effective and the disease still results in complete paralysis and death within the first 5 years after diagnosis [31, 32].

3.2 Troponin variation in cardiomyopathy

The calcium-mediated interaction between actin and myosin is controlled by cardiac regulatory proteins, cardiac troponin T (cTnT) and troponin I (cTnI). The cardiac forms of these regulatory proteins theoretically have the potential of being unique to the myocardium [34], as they are coded for by specific genes.

Cardiac troponins are detected in the serum by the use of monoclonal antibodies to epitopes of cTnI and cTnT. These antibodies are highly specific for cardiac troponin and have negligible cross-reactivity with skeletal muscle troponins. Indeed, cTnI has not been identified outside the myocardium [34]. Cardiac troponin T is expressed to a small extent in skeletal muscle; however, the current cTnT assay does not identify skeletal troponins [35].

The majority of cTnI and cTnT form part of the contractile apparatus within the myocardial cell with lower concentrations found in the cytoplasm [35]. Whenever there is myocardial ischemia resulting in myocardial necrosis, the cTn will be released from the cytosolic pool into the bloodstream within a few hours of the injury. This is typically followed by a more prolonged and sustained elevation of cTn due to degradation of the contractile apparatus, which may also be a reflection of the size of the infarct [35].

However, the release kinetics of cTn after the myocardial injury can differ between individuals and is also dependent on myocardial blood flow. It can also differ between cTnI and cTnT which are thought to have monophasic and biphasic concentration-time profiles respectively, and with the increase in cTnT tending to last for longer than that of cTnI [34].

After the onset of an acute coronary event, cardiac troponins may not be detected in the serum for up to 4 hours and should be repeated 12 hours after the first test, if the troponin concentration is not raised in an individual presenting with chest pain.

In the identification of cardiac muscle damage, the measurement of serum cTnI and cTnT are superior in terms of sensitivity and specificity to cardiac muscle enzyme measurements [36]. Elevated cardiac troponin concentrations are now an acceptable standard biochemical marker for the diagnosis of myocardial infarction [37].

In order to enhance the comparison of results for cTnT, from one laboratory to another, troponin T is measured using a single assay, and a cutoff value of 0.1 μg/liter is indicative of myocardial damage [38]. However, there are several cTnI assays with different sensitivities and cutoff values. According to the European Society of Cardiology and American College of Cardiology consensus criteria, serum cTnI values that indicate myocyte necrosis/myocardial damage range from 0.1 to 2 μg/liter [38].

In the management of patients with acute chest pain, the measurement of cardiac troponins as markers of myocardial damage has produced two important

beneficial effects on clinical practice [39]. The first beneficial effect is that more patients with chest pain who would not have been diagnosed as having myocardial damage with conventional muscle enzyme assays are being diagnosed with myocardial infarction, even in the absence of ST-segment elevation. The second beneficial effect is that mortality is reduced because many of these patients are at high risk of full-thickness myocardial infarction or even death within 6 month period [40, 41].

The *Universal Definition of Myocardial Infarction* requires at least one cTn concentration above the 99th percentile value of a normal reference population for the diagnosis of myocardial injury [38]. However, there have been some concerns regarding the use of a 99th percentile threshold value for hs-cTn because of its limitations [42]. Firstly, the 99th percentile varies with assay [43]. Secondly, the 99th percentile varies with reference population selection (age, gender, ethnicity, and definition of healthy status), reference population size, and the statistical method used to calculate it [44, 45]. Some studies have shown that elevations of hs-cTn can be seen in older adults, which may be independent of pathological conditions [46, 47]. Thirdly, detectable chronic elevations in cTn above the 99th percentile are commonly seen in conditions such as chronic renal or cardiac failure [48, 49]. In addition, the improved analytical sensitivity of these assays has resulted in the detection of elevated cTn in numerous cardiac and non-cardiac conditions that cause myocardial cell necrosis, such as myocarditis, arrhythmia, cardiac procedures, pulmonary embolism, and sepsis [34, 41]. Due to these challenges, international guidelines have sought to promote consistency by proposing recommendations for determining 99th percentiles [50, 51]. It would therefore seem that the 99th percentile should not be the only metric for diagnosing acute myocardial injury.

Cardiac troponins may also be elevated in many other conditions associated with secondary ischaemic injury [44], such as large pulmonary emboli, coronary spasm, cardiac arrhythmias [52], hypertrophic cardiomyopathy [52], idiopathic dilated cardiomyopathy [53, 54]. It can also be elevated in conditions that cause myocardial injuries, such as cardiac trauma, chemotherapy [55], myopericarditis [55, 56], septicemia [57].

Some studies also found that cTn was detectable in nearly all children, where concentrations increased with increasing age and left ventricular mass, thus supporting the notion that cTn release is not always pathological [58].

In addition, it has recently been demonstrated that cTn may exhibit diurnal variations [59, 60]. One study noted that cTnT concentrations exhibited a decreasing trend between morning and afternoon (0830 hours and 1430 hours) for healthy individuals and individuals requiring hemodialysis [59]. For cTnI concentrations, a decreasing trend during these hours was also noted in individuals requiring hemodialysis, however, the pattern was not apparent in healthy individuals [59]. Furthermore, another study in men with type 2 diabetes found that cTnT decreased during the day and then increased during the night, with peak concentrations in the morning at 0830 hours [58]. This was further confirmed in another study of healthy individuals, where cTnT exhibited diurnal variation but cTnI did not have such variation [60]. In other words, cTn can be described as organ-specific but not disease-specific.

4. RNA splicing in disease diagnosis

RNA splicing is a post-transcriptional process necessary to form a mature mRNA [61]. There are two main forms of splicing, that is, constitutive splicing and alternative splicing.

Constitutive splicing involves removal of introns from the pre-mRNA and joining the exons together to form a mature mRNA. Alternative splicing describes how exons can be included or excluded in different combinations to create a

diverse array of mRNA transcripts from a single pre-mRNA and therefore serves as a process to increase the diversity of the transcriptome. It was initially thought that about 5% of human genes were subjected to alternative splicing [62]. Now, after the implementation of next-generation sequencing technologies, it is now known that the vast majority, >95% of mRNAs, are subjected to alternative splicing [63]. However, the function of a large fraction of these splice isoforms is still unknown.

Splicing is more prevalent in multicellular than in unicellular eukaryotes because of the lower number of intron-containing genes in the latter [64]. As evolution progress, alternative splicing becomes more prevalent in vertebrates than in invertebrates. Skipping of a single exon in the RNA-binding protein (RBP) and polypyrimidine tract binding protein 1 (PTBP1) may be responsible for numerous alternative splicing changes between species, which suggest that one splicing event can augment the varieties observed in transcriptome between species [65].

The hypothesis that alternative splicing largely contributes to organism diversity is fueled by the observation that the total number of protein-coding genes does not differ much between species. And indeed, as we move up the phylogenetic tree, alternative splicing complexity increases, with the highest complexity in primates [66, 67].

4.1 Major and minor spliceosome

RNA splicing is performed by the spliceosome, a large and dynamic ribonucleoprotein complex composed of proteins and small nuclear RNAs (snRNAs), which assembles on the pre-mRNA (**Figures 1** and **2**).

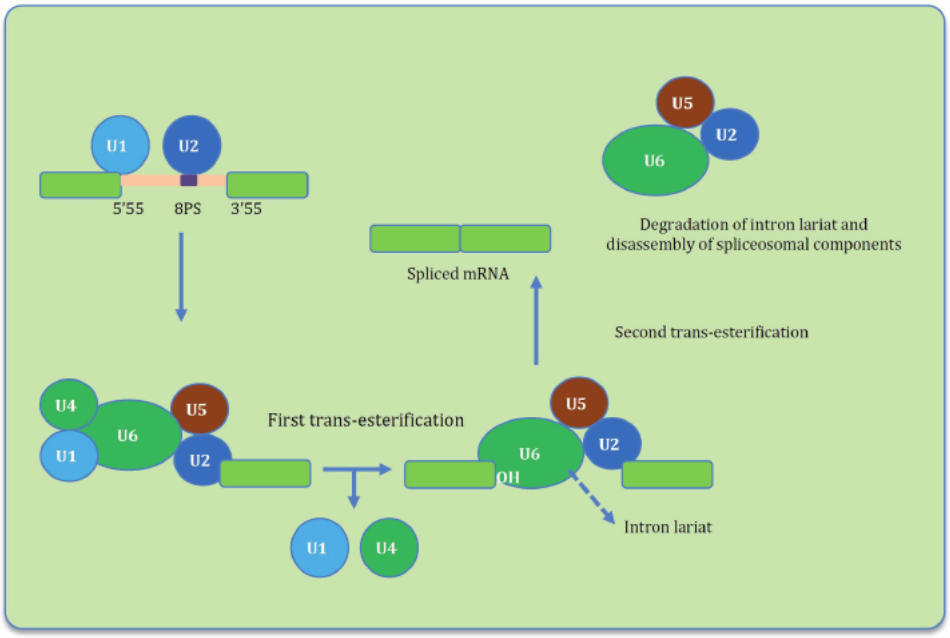

Figure 1.
Two-step splicing reaction. Splicing occurs by a 2-step trans-esterification reaction to remove introns and join exons together. The first step, U1 small nuclear ribonucleoprotein (snRNP) assembles at the 5′ splice site of an exon and U2 snRNP at the branch point sequence (BPS), just upstream of the 3′splice site of the adjacent/downstream exon. This configuration is known as the pre-spliceosome. Hereafter, U1 and U2 are joined by the snRNPs U5 and U4–U6 complexes to form the pre catalytic spliceosome. Next, U4–U6 complexes unwind, releasing U4 and U1 from the pre-spliceosomal complex. This allows U6 to base pair with the 5′ splice site and the BPS. The 5′ splice site gets cleaved, which leads to a free 3′ OH-group at the upstream exon, and a branched intronic region at the downstream exon called the intron lariat.

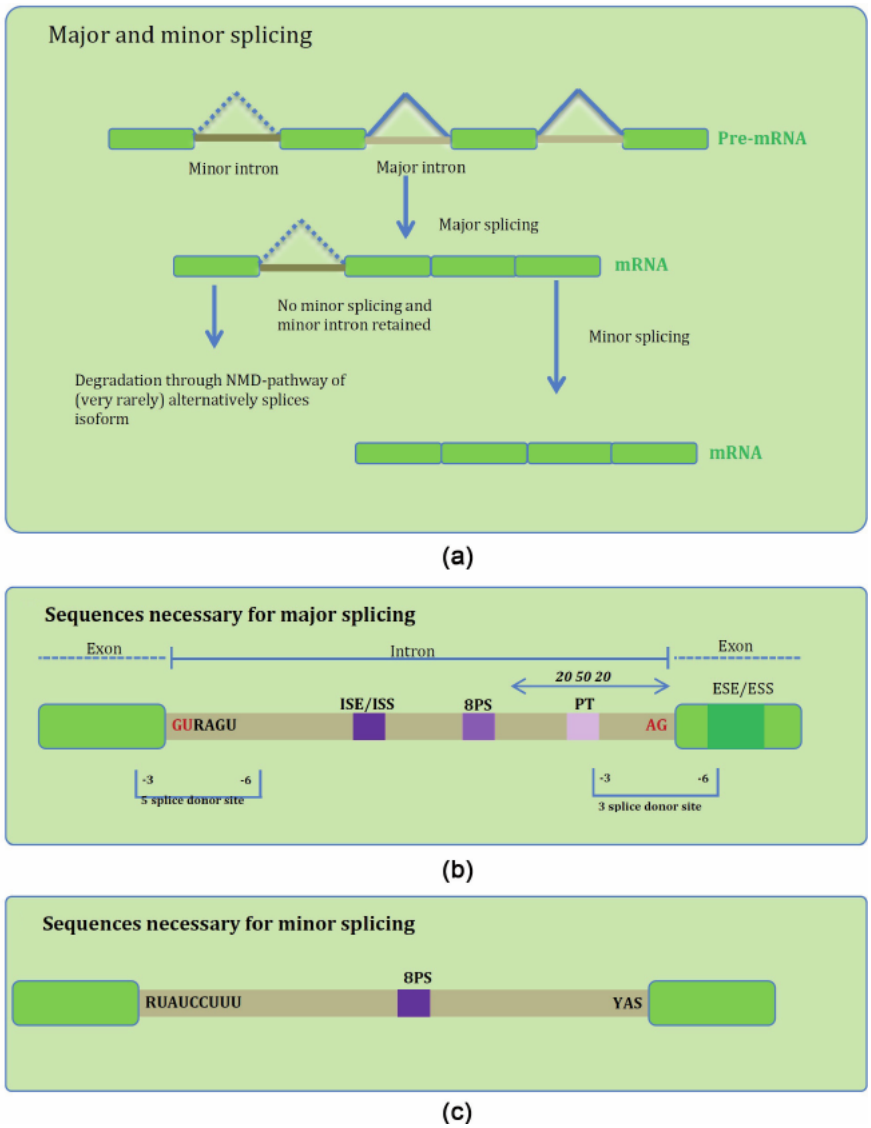

Figure 2.
*Major and minor splicing. (A) Major, and minor splicing. The major introns are spliced out, and minor introns are either retained (and the mRNA is most often subsequently degraded) or the minor intron is spliced out, and a mature mRNA is formed. (B) The 4 basic splicing signals are the 5′ splice donor site, the 3′ splice acceptor site, the branch point sequence (BPS), and the polypyrimidine tract (PT). Spliceosomal components recognize and bind to these sequences and mediate the splicing reaction. Intronic and exonic splicing enhancers and silencers determine the inclusion rate of exons. The BPS (major, YNYURAY; minor, UCCUUAACU) is located 20–50 bp upstream of the 3′ splice site, and the PT (Y10–12) is located in between the BPS and the 3′ splice site (N=any nucleotide, Y=C or U, R=A, or G and S=C or G). (C) Minor splicing uses different 5′ and 3′ splice sites and BPS, and lacks the PT. ESE indicates exonic splicing enhancers; ESS, exonic splicing silencers; ISE, intronic splicing silencers; and ISS, intronic splicing silencers. Note: The **Figures 1** and **2** are a modification from van den Hoogenhof et al. [68].*

During the second step, U5 pairs with sequences in both the 5′ and 3′ splice sites, positioning the 2 ends together. The 3′ OH-group of the upstream (5′) exon fuses with the 3′ intron-exon junction, thereby conjoining the 2 exons and excising the intron in the form of a lasso-shaped intron lariat. Finally, the spliceosome disassembles, and all components are recycled for future splicing reactions.

Recent evidence has shown that splicing does not occur after transcription, but happens during transcription; therefore, the vast majority of human introns are spliced out when transcription is still taking place [69].

4.2 RNA splicing in cardiomyopathy

Several mouse models suggest a role for splicing factors in postnatal heart development. One such example is the alternative splicing factor ASF/SF2 (or SFRS1), an SR protein that is ubiquitously expressed and acting as an alternative splicing regulator [70]. ASF/SF2 conditional knockout mice die 6–8 weeks after birth, due to hypercontractile cardiac phenotype caused by a defect in Ca^{2+} handling. When ASF/SF2 is deleted, it leads to mis-splicing of several genes, including cardiac troponin T (cTnT), LIM-domain binding 3 (LDB3), and Ca^{2+}/calmodulin- dependent protein kinase (CamkIIδ), Atypical alternative splicing of CamkIIδ, cTnT, and LDB3 can present 20 days after birth, even though ASF/SF2 was deleted at the early stages of cardiogenesis.

Mis-splicing of CamkIIδ in ASF/SF2 knockout hearts can lead to perturbation of Ca^{2+} handling and severe excitation-contraction coupling defects, which in turn leads to dilated cardiomyopathy (DCM).

Embryonic lethality may occur in systemic deletion of SC35 in mice, even before the onset of cardiogenesis [71]. Attempt to bypass this problem by generating a heart-specific knockout of SC35 uncovered the role of SC35 in the heart, as cardiac hypertrophy and DCM developed in these mice at 5–6 weeks of age [71].

In conclusion, ablation of SC35 in the heart shows that proper expression of this splice factor during postnatal heart development is essential to maintain cardiac form and function.

Severe and lethal DCM has been reported to occur 2 weeks after birth in mice with deletion of hnRNP U in the mouse heart [72]. The importance of alternative splicing of Ca^{2+}-handling genes in early postnatal heart development can be observed in the role of heterogeneous nuclear ribonucleoprotein U (HnRNP U) in splicing of calcium/calmodulin-dependent protein kinase IIδ (CamkIIδ).

4.3 Role of alternative splicing in disease phenotype

Atypical alternative splicing has been documented to contribute to disease severity and susceptibility [73]; as observed in retinitis pigmentosa, Prader-Willi syndrome, and spinal muscular atrophy [74, 75]. Spinal muscular atrophy, for example, is caused by the loss of the survivor of the motor neuron-1 (SMN1) gene, which is required for proper assembly and transport of snRNP [74].

Kong et al. [76] used a genome-wide approach to study alternative splicing changes in the diseased heart. The splicing of 4 key sarcomeric genes, troponin T (TNNT)-2, TNNI3, MYH7, and FLNC, were significantly altered in human ischemic cardiomyopathy, DCM, and aortic stenosis.

5. Epigenetic DNA modifiers

Epigenetics and its attendant markers influence the proliferation of diseases and their phenotypes. Outside, DNA canonical structure, DNA folds into alternative structures including DNA hairpins, cruciforms, triplexes or G-quadruplexes (G4), and holiday junctions [77, 78]. Besides these DNA structural changes, epigenetics processes, using DNA methylation and histone modification as the driver, are another primary vehicle for changes in DNA. Changes due to epigenetics modification with time can alter our phenotypes profoundly. Known facts are that everything from what we eat, drink, and smoke to other factors within our immediate environment including, stress can interfere with the way our genes express themselves up and down the line with the finest totality [79]. The primary

vehicles for epigenetic changes are DNA methylation and histone modification; there are many known enzymes that act on histone modifications by either adding or removing the covalent modifications. Such changes influence the degree of interaction between DNA and histone, which have some profound effects on the ability of that DNA to be transcribed. Histone modifications are subject to rapid changes (in seconds/minutes), giving room for the cell to respond to external stimuli. Furthermore, many of the known enzymes responsible for modifying histone residues have numbers of non-histone substrates such as transcription factors [80, 81].

Some mechanisms for the function of histone modifications have been characterized including; the compression of chromatin, and the recruitment of non-histone proteins [82]. There are different types of modification and these determine the amino acid residue produced. The modifications of histone lead to either gene activation or repression, and the addition of acetyl groups, to the tail of histone H3, neutralizes the basic charge of the lysine, leading to the unfolding of the chromatin, allowing transcription to occur. Conversely, the removal of these acetyl groups results in chromatin compression, which prevents transcription [82]. These kinds of changes in chromatin structure help to prevent access by other proteins that can further modify the chromatin (e.g., remodeling ATPases).

Understanding the etiology of some of these diseases, from PPI, protein DNA/RNA interaction is important as it will herald in more robust drug treatments for patients

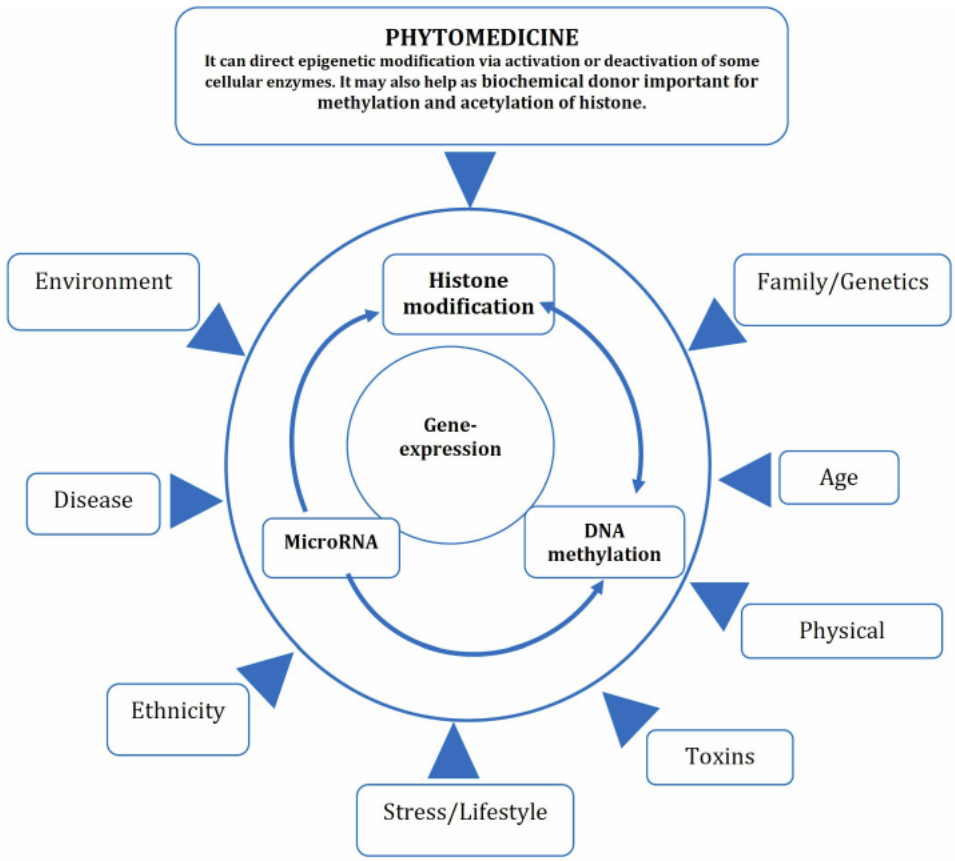

Figure 3.
Factors influencing epigenetics (modified from [84]): complex interplay is required for a wholesome gene expression understanding; such complexes will further enhance the use of phytomedicine in disease management.

with specific disease phenotypes. Along this line, Okoh et al. [81] recently, using available data, espoused the need for the combination of herbal medicine to target some epigenetic markers by way of epigenetic engineering (site-specific DNA binding module fusions with DNA demethylating enzymes for epigenetic induction of for instance; fetal hemoglobin (HbF) for therapy of sickle cell disease (SCD)). This is in consonance with earlier postulation [83, 84], implying such technique may provide a better way to activate/or repress inherent gene expression, bearing transient modification of DNA and histones should remain stable over many cell divisions helping in delaying HbF switching [83, 84].

Moreover, Okoh et al. [81], suggested that the de-methylation of DNA at the CpGs site on both DNA strands may be possible using the combination of herbal medicine, foods rich in flavonoids could be vital in tweaking histone acetylation, which can modulate gene expression. The figure below postulates the complex interplay between, epigenetics and phyto-compound modifiers towards enabling gene transcription for proper protein translation (**Figure 3**).

6. Future perspective

CRISPR/Cas9-based therapy, are been used as a candidate to be administered systemically, via intravenous infusion, for precision editing of a gene in target tissue in humans [85]. Similarly using this technology, gene therapy was developed to treat the rare neurodegenerative condition, Dopamine Transporter Deficiency Syndrome (DTDS) using a personalized approach with a view to counter the exact genetic fault present in a patient's neurons [85]. Using a novel approach where, skin cells from patients, turned into pluripotent stem cells in the laboratory with the aim to get neuronal cells with the disease-causing mutation. A vector carrying adeno-associated virus gene therapy was created to target the neurological fault and its efficacy was tested in both neuronal human cell lines and a mouse model, with the corresponding loss of function mutations in *SLC6A3* [85]. This research ingenuity/approach has provided, promising results leading to some clinical trials that may put an end to this cruel disease.

DTDS is an area of unmet medical needs and the disease is also known as infantile parkinsonism-dystonia, due to it having neurodegenerative and movement symptoms similar to Parkinson's disease [85]. It is a very rare inherited condition known to affect around 50 children around the world. Although this might be due to under-diagnosis by clinicians bearing the symptoms are similar to other inherited movement disorders e.g., cerebral palsy [85].

Environmental factors are implicated in the formation of ROS affecting human health by directing epigenetics signature of the genome, such could also drive the addition of methyl group (–CH3) to some nucleotides neighboring guanosine (CpG islands) of the genome. These are areas where drug repurposing becomes essential as they could target methylation processes which are amongst, inherent biochemical/epigenetics machinery of cells, containing necessary pathways that allow environmental agents to induce mutations. Bearing these epigenetic signatures play a significant role in genomic balance, they play a leading role in several diseases hence are the essential target for drug repurposing.

Many diseases present some inherent opportunities via epigenetics markers that required intelligent manipulation of phyto-compounds to access new therapy that is efficient and easily accessible. Phytochemicals are known to play vital roles in preventing oxidative stress with concomitant damages [2, 85]. At the cellular and molecular level, they inactivate Reactive Oxygen Species (ROS). And under specific low concentration, inhibit or delay oxidative processes by interrupting the radical

chain reaction of lipid peroxidation [2, 86]. Bioactive components with anti-oxidative capacity naturally present in food are of great interest due to their beneficial effects on human health as they offer protection against oxidative deterioration.

DNA processes such as replication, transcription, recombination, and repair are, known to be facilitated by several factors covered in this chapter and others such as supercoiling that help facilitate both the packaging of DNA and many fundamental genetic processes that enabled the enzymatic manipulation of DNA. Aberrant RBP-RNA interactions are now known to promote disease progression, as much as mutations in TFs. RBP's role in disease was initially understudied because of their systematic evaluation was limited by, lack of sensitive and efficient assays for phenotypic interrogation of individual RBPs.

There is profound evidence that suggests, consumption of food rich in phytochemicals may progressively reduce the risk of different diseases by modulating immune-inflammatory markers [87]. Using the combination of disparate molecular/biophysical tools we recently [88], compared the binding affinity of artesunate and azadirachitin to gephyrin E this is towards enabling insights into natural bioactive compounds useful for rational drug design, essential in the race to manage myriad of disease phenotypes. The results from our research and others are necessary as they, may provide, the impetus for more studies into bioactive components of plant origin towards the effective management of different disease phenotypes.

6.1 Next-generation sequence in disease diagnosis

Next-generation sequencing (NGS), is a massively parallel and a high-throughput DNA sequencing technology that enables the fast generation of data on thousands to millions of base pairs of DNA from an individual patient by sequencing large numbers of genes in a single reaction [89]. NGS can sequence millions of DNA fragments in a massively parallel fashion, instead of sequencing a single DNA fragment one at a time, as observed in traditional capillary electrophoresis sequencing. The general workflow of NGS includes four main steps:

I. library preparation,

II. cluster generation,

III. sequencing, and

IV. data analysis.

Sequence reads are produced from fragment libraries, a pool of adaptor-ligated and enriched DNA fragments. One advantage is that a small quantity of DNA, from a patient, is needed to produce a library.

In step 1, patient DNA is randomly fragmented by different methods and then prepared for sequencing by ligating specific adaptor oligonucleotides to both ends of each DNA fragment. Adapter-ligated fragments are further enriched with specific oligonucleotides designed for the target genes included in the NGS panel and are then amplified by polymerase chain reaction (PCR). The prepared library is loaded into a flow cell for cluster generation and subsequent sequencing.

During sequencing, short read lengths (35–250 bp, depending on the platform) sequences that are produced are then aligned to a reference genome with bioinformatics software [89].

During data analysis, variant calling can be achieved by various standard and in-house analysis pipelines. All detected variants are checked against standard databases (e.g., dbSNP137, 1000 Genomes Project, Exome Variant Server, ExAC Browser, OMIM catalog, ClinVar, Human Gene Mutation Database) to enable interpretation of the pathogenicity of a given variant.

Next-generation sequencing panels are now commonly used in clinical diagnosis to identify genetic causes of various monogenic disease groups, such as epilepsy [90], intellectual disability [91, 92], neurodevelopmental disorders [93], neuro-metabolic disorders [94], amongst others.

The use of NGS in clinical laboratories is increasing, with application in the diagnosis of immune disorders, infectious diseases, human hereditary disorders, in non-invasive prenatal diagnosis, and recently, in the therapeutic decision making for somatic cancers [95, 96].

Today two different NGS technologies are mainly used in clinical laboratories: Ion Torrent and Illumina systems [97].

The Ion Torrent exploited the emulsion PCR using native dNTP chemistry that releases hydrogen ions during base incorporation by DNA polymerase and a modified silicon chip detecting the pH modification [98], while Illumina technology is based on the existing Solexa *sequencing by synthesis* chemistry with the use of very small flow-cells, reduced imaging time and fast sequencing process [97].

6.2 Usefulness of NGS

NGS approaches will remain useful because:

1. It is highly accurate and cost-effective.

2. It has a wide application for use in clinically heterogeneous inherited disorders, resulting in an increase in the number of reported disease-causing genes.

NGS is appealing when there is a genetic contribution in heterogeneous and complex diseases, such as in cardiomyopathies, in cardiac arrhythmias, in connective tissue disorders, in mental retardation or autism, and where a large number of genes are involved in a large phenotypic syndrome [99, 100]. In these cases, NGS approaches allow us to test a large number of genes simultaneously in a cost-effective manner [101].

Two options of NGS are currently available [101]:

1. Targeted gene panels sequencing or

2. Whole-exome sequencing (WES).

Targeted sequencing is applicable for genetic disorders, such as non-syndromic deafness [98], common diseases, such as hypertension and diabetes [102], or in traditional cytogenetic and Mendelian disorder diagnosis [103]. The main limitation of targeted sequencing is the rigidity of testing only a selected number of genes. Since the genetic field is rapidly evolving, new genes may be associated with a clinical phenotype, and as such redesigning and revalidation of the panel is needed [101].

The WES application could be applicable for the identification of genes responsible for the dominant Freeman-Sheldon syndrome, the recessive Miller syndrome, and the dominant Schinzel-Giedion syndrome [104]. The shortcoming of WES is

that about 10% of targeted bases sequenced in WES do not get the 20 read depth [105], required for clinical confidence and interpretation, and approximately only 85% of genes associated with human diseases into the principle database (OMIM) receive the adequate coverage [106].

6.3 Challenges of NGS in disease diagnosis

In the NGS process, one limiting step is the complexity of genetic variation interpretation in whole-exome, due to the presence of thousands of rare single nucleotide variations without pathogenic effect. Moreover, in the majority of human diseases, the pathological phenotype may be caused by a pathogenic rare mutation with a strong effect or it may be caused by a co-presence of multiple genetic variations [107].

Another important challenge of the use of the NGS approach in clinical diagnostic is the management of the amount of data generated [108]. Indeed generation, analysis, and also storage of NGS data require sophisticated bioinformatics infrastructure [109], which could be capital intensive.

A skilled bio(chem)-informatics staff is needed to manage and analyze NGS data, therefore increasing the impact of computing infrastructure and manpower on costs of NGS applications in clinical diagnostics [110, 111].

7. Conclusions

The interest in studying protein interactions, their variations, and their constituent's effects on pathological conditions has grown within the last few decades. These interests are behind, for instance, the increasing examination of the application of mass spectrophotometer (MS)-based experimental analyses of model systems to explore heterogeneous PPI networks and protein complexes, which will promote drug repurposing within the context of human diseases.

The use of other robust technology such as NGS in the study of biological processes, which would have otherwise remained elusive, is the driver for the actualization of personalized medicine for which drug repurposing form a central aspect. Molecular interactions at both the DNA-RNA/protein level are sequined with the SNP, epigenetics, mutations causing variants, and other factors emanating from our immediate environment e.g. stress. Technological advancement discussed in this chapter is all part of the process for science and scientists to understand the biological phenomenon that governs life at the molecular level.

Many disease variants resulting from SNP, single point mutation, and RBP role in disease manifestation are discussed with a view to heighten our thinking towards drug repurposing, the uptake of phytomedicine, and bioactive compounds in the management of various disease phenotypes.

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Chapter 15

Repurposing Market Drugs to Target Epigenetic Enzymes in Human Diseases

Aishat Motolani, Matthew Martin, Steven Sun and Tao Lu

Abstract

Drug discovery is an exciting yet highly costly endeavor. In the United States, developing a new prescription medicine that gains marketing approval takes near a decade and costs drugmakers for near 3 billion. More challengingly, the success rate of a compound entering phase I trials is just slightly under 10%. Because of these mounting hurdles, repurposing market approved drugs to new clinical indications has been a new trend on the rise. Another merit to this approach is the already confirmed toxicity profiles of the drugs and their possession of drug-like features. Thus, repurposed drugs can reach the market approved stage in a much faster, cheaper, and more efficient way. Notably, epigenetic enzymes play a critical role in the etiology and progression of different diseases. Researchers are now assessing the possibilities of using market approved drugs to target epigenetic enzymes as a novel strategy to curtail disease progression. Thus, in this book Chapter, we will provide an outlook on repurposing market drugs to target epigenetic enzymes in various diseases. Consequently, this book Chapter will not only provide the readers with current knowledge in this specific field, but also will shed light on the pathway forward for repurposing market drugs to target epigenetic enzymes in human diseases.

Keywords: disease, drug, EMA, epidrug, epigenetic, FDA, repurposing

1. Introduction

1.1 Overview of drug approval agencies

Drug approval agencies are responsible for the oversight and scientific evaluations that ensure the safety and effectiveness of the drugs that reach the market, and eventually, patients. Several agencies regulate drug approval worldwide, with the United States (US) and Europe being the top regulators. Some examples include the US Food and Drug Administration (FDA), the European Medicines Agency (EMA), Health Canada, Japan's Pharmaceutical and Medical Devices Agency (PMDA), Australia's Therapeutic Goods Administration (TGA), and so on [1]. In the last 5 years, the US FDA has approved about 245 drugs, many of which include anti-cancer and neurological disorder drugs [2]. Following drug discovery and preclinical trials, different types of applications can be filed to the FDA to begin a drug's journey to the market. A sponsor can either file the Investigational New Drug (IND) application followed by the New Drug Application (NDA) or the Abbreviated New Drug Application (ANDA). An IND application is submitted if

a drug is deemed safe after preclinical investigations. Then, an NDA can be submitted after the drug is deemed safe from the clinical trial results. At this point, a request can be made to produce and market the drug in the US [3]. On the other hand, ANDA is filed for the approval of generic drugs. Although clinical studies are not needed for this application, sponsors must prove that their drug is similar and bioequivalent to the original approved branded counterpart [4]. In the European Union, sponsors submit a clinical trial application (CTA) followed by a marketing authorization through either a centralized process or a decentralized process [3, 5]. Notably, both the FDA and EMA have similar yet distinct regulatory mechanisms to categorize drug approvals. For example, the FDA may grant drugs a standard approval, fast-track designation, accelerated approval, breakthrough designation, or priority review. Similarly, besides the standard approval, the EMA has accelerated assessment and conditional approval for expedited programs to bring a drug to market faster [5]. Taken together, these drug approval agencies do participate in a global collaborative effort to protect and improve public health by ensuring patients' timely access to safe and effective medicines [6].

1.2 Overview of traditional drug discovery method

The world population is constantly increasing and aging, with a census of close to 8 billion people in 2021 [7]. Additionally, there remains a growing necessity for novel therapeutics to combat the increasing number of cancers, metabolic disorders, infectious diseases, neurodegenerative diseases, and diabetes, as they are a major burden on public health. Despite this necessity, the rate of creation and approval of novel therapeutics is slow by comparison with estimated costs ranging from several hundred million dollars (USD) to several billion per therapeutic with an estimated development time of 12–15 years [8]. The reasons behind these high costs and difficulties with bringing novel therapeutics to market are in some ways straightforward: many projects fail in clinical trials; clinical trials are expensive and time-consuming; therapeutics have failed in the market due to previously unknown public health concerns; research costs are constantly increasing, and the initial investment cost of each therapeutic is high for a pharmaceutical company [9–12]. Traditional drug discovery involves the identification or creation of a new molecular entity (NME). The identification process of an NME usually proceeds as follows: initial basic research generates data supporting inhibition or activation of a protein or pathway that will result in a therapeutic effect in a disease state. Then a lead discovery compound such as a small molecule or biological therapeutic is discovered following some compound screening. The target is validated, and preclinical screening is performed, then the therapeutic can go through clinical trials before filing for drug agencies' approval [11]. The basic steps of this process are illustrated in **Figure 1**. However, at any step of the drug discovery timeline, the therapeutic can fail for a multitude of reasons. Generally, it comes down to two main factors: efficacy and safety [11]. For instance, several therapeutics of AstraZeneca have failed in phase II trials due to toxicological concerns [13]. Other studies stopped clinical trials when the newly developed therapeutics had decreased efficacy compared to existing therapeutics [14]. Furthermore, the time during each of these steps can be lengthy with several years, such as effort needed for compound discovery, clinical development, clinical trials, and FDA or EMA filing (**Figure 1**) [15]. For instance, the overall percent likelihood of approval (LOA) from phase I to approval in all therapeutic fields from 2011 to 2020 was merely about 8% [16]. The LOA differed greatly per therapeutic and per phase (lead compound to phase I, phase I to phase II, etc.). The only step of drug discovery where therapeutics were highly likely to progress was during NDA approval, with a success rate of 80–90% [17]. These

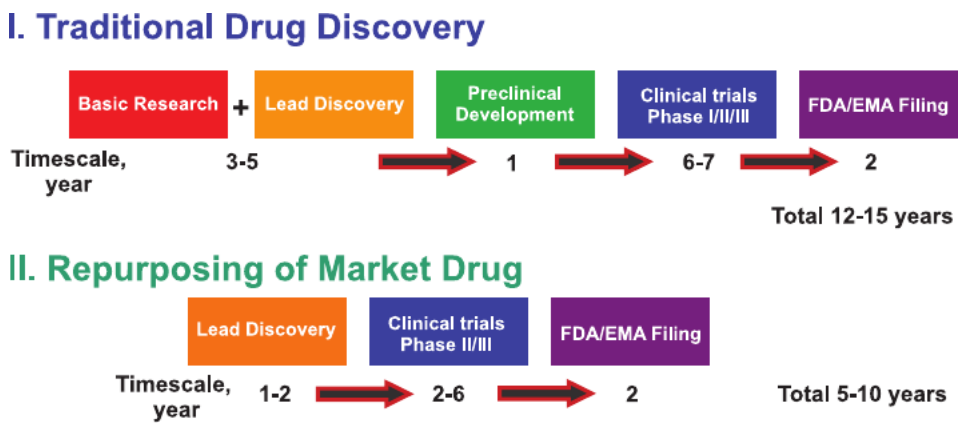

Figure 1.
Traditional drug discovery process (I) from discovery of target, validation, trials, and FDA/EMA approval with timescale versus repurposing of a market drug (II). This schematic describes the timeline of traditional drug discovery, wherein years of rigorous basic research leads to discovery of a drug, which then undergoes extensive pre-clinical development before going through the clinical trial and market approval phase. With the advent of drug repurposing approach, the timeliness and cost-efficiency of drug discovery significantly improved the driving of a drug for newer indications to the market faster.

are what led to the long development times for therapeutics discovered through traditional drug discovery methods. In summary, the overall takeaway message is that there is a multitude of factors that can stall drug discovery, and an alternative methodology may be a better approach to bring therapeutics to market.

1.3 Overview of basis of drug repurposing

Due to the immense financial costs and timescale associated with traditional drug discovery methods, it is natural to assume alternatives may be preferable, such as repurposing current therapeutics. As shown in **Figure 1**, repurposing known therapeutics follows a similar but truncated development cycle as traditional drug discovery. The timeline includes the discovery of a therapeutic target, usually a new therapeutic indication for a previously approved indication. Then, it is followed by clinical trials. Given that the information on the preclinical, pharmacokinetic, and pharmacodynamic are already known, the clinical trial phase moves faster. Following this step is filing for market approval as usual [18, 19]. This allows for a development time of 5–10 years compared to 12–15 years of development for traditional drug discovery (**Figure 1**). This also drives down the two major factors hampering novel drug development: cost of new therapeutics and time of discovery to market. Therefore, it is not surprising that an increasing number of therapeutics developed by the FDA or EMA are repurposed therapeutics [20]. Interestingly, the discovery of novel indications for therapeutics has been made through a multitude of approaches. Drug repurposing involves integrating data from multiple resources and the use of different approaches to allow for the discovery of novel indications. One major path for novel indications is model-based computation or *in silico* drug repurposing. This can include numerous screens of a therapeutic concerning its drug molecular targets, chemical structure, and signaling pathways to predict unknown targets or biomarkers for disease [18]. Besides computational modeling, high-throughput and/or high-content screening (HTS/HCS) of drug compounds are frequently used to screen known therapeutics for novel targets [21]. Additionally, *in silico* screening of known compounds can be used for molecular docking or binding-based studies [22]. Furthermore, *in silico* screening can also help with the computational prediction of novel metabolic pathways, signaling pathways,

and protein-protein interactions between diseases and known drugs [23]. Other major methods of screening for novel indications of current therapeutics include recently AI-based machine learning, which has been used to some effect to screen large groups of compounds for anti-Sars-Cov-2 inhibition [24]. Further predictive modeling of drug repurposing includes network modeling wherein networks of drugs, genes, and drug products, as well as their interactions and relationships can be modeled, allowing for a greater understanding of structure-guided targeting of therapeutics [25]. Furthermore, large-scale genome-based predictive modeling, such as genome-wide association studies (GWAS) can help predict potential novel therapeutic interactions [26]. Another aspect not commonly studied is the known side effects of therapeutics and how it can be used to identify novel therapeutic targets *via* computational modeling [27]. According to Sahragardjoonegani and colleagues, roughly two-thirds of new therapeutics approved by the FDA within the last 15 years have not been indicated for secondary indications besides their original purpose [28]. This creates a largely untapped field of current therapeutics that have not been studied in the context of other diseases. Overall, drug repurposing for therapy requires less time and cost for development and research than traditional drug discovery. Thus, it is an attractive approach for the discovery of new therapeutics.

2. Role of epigenetic enzymes in human diseases

Epigenetics is the study of mechanisms that results in heritable changes in gene expression without the alteration of the genetic code [29]. The deregulation of epigenetic mechanisms, such as DNA methylation and histone modifications, have been reported to facilitate differential expression of genes, many of which underlie the etiology and/or the progression of human diseases [30].

These epigenetic mechanisms are mediated by their respective epigenetic enzymes. For instance, DNA methyltransferases (DNMTs) coordinate the methylation of DNA by catalyzing the transfer of a methyl group to cytosine (C) from the donor molecule S-adenosylmethionine (SAM) [31]. The methylated DNA is read by methyl-Cp-guanine (G) binding domains (MBD) protein. DNA methylation can be reversed by a group of human demethylase enzymes termed ten-eleven translocation proteins (TET 1/2/3) [31]. DNA methylation is responsible for gene silencing and often occurs in regions rich in C and G nucleotides, also known as CpG islands. The catalysis of DNA methylation is primarily conducted by the following family of DNMTs: DNMT1, DNMT3A, and DNMT3B [32]. These enzymes help maintain the integrity of the human genome, regulate transcriptional processes, and aid cellular development and differentiation [33]. Thus, dysregulation of DNA methyltransferase and demethylases are implicated in several human diseases.

Similarly, the covalent modification of histones is another facet of epigenetics that plays a pivotal role in human diseases. The core histone proteins, H2A, H2B, H3, and H4, form an octameric structure that wraps about 146 base pairs of DNA to form a nucleosome, and the linker histone, H1, connects the repeating nucleosomes that make up the chromatin. Histones' terminal regions project out of the chromatin in a tail-like structure, and these tails are subjected to post-translational modification (PTM) by different histone-modifying enzymes [28, 31]. Some of the most common classes of histone-modifying enzymes include histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs) [34]. HATs and HDACs are writers and erasers of acetylation, respectively, on lysine (K) residues of histones and non-histone proteins. The acetylation of histones results in a relaxed chromatin that promotes gene transcription [35]. HATs are classified into Type A: p300/CBP, general control

non-depressible 5 (GCN5)-related N-acetyltransferase (GNAT), Moz, Ybf2/Sas3, Sas2, Tip60 (MYST), nuclear receptor coactivator- (NCOA-) related HAT, and transcription factor-related HAT; and Type B: HAT1, HAT2, HatB3.1, Rtt109, and HAT4 [36]. While HDACs comprise 18 enzymes: HDAC1-11 and sirtuins (SIRT)1-7 [37]. The BRD and extra terminal domain (BET) proteins are responsible for recognizing K residues that are acetylated [37]. On the other hand, methylation of histones, which occurs on either K or arginine (R) residues of histones, can lead to gene transcription repression or activation. The addition of methyl group(s) to histones is mediated by HMTs while its removal is mediated by HDMs [34]. HMTs are further subdivided into lysine methyltransferases (KMTs) and arginine methyltransferases (PRMTs) [38]. Similarly, HDMs are classified into lysine demethylase 1 (LSD1 or KDM1) and Jumonji C (JmjC) domain-containing histone demethylases [39]. Together, these classes of histone-modifying enzymes regulate the expression of genes vital to many human biological processes.

2.1 Epigenetic enzymes implicated in cancer

The dysregulation of epigenetic enzymes is one of the chief contributors to cancer development and progression. Several cancers are accompanied by significantly altered DNA methylation status, and this has been shown to serve as a diagnostic and prognostic marker [40]. The resulting imbalance in gene expression is mainly caused by hypomethylation of oncogenic genes or hypermethylation of tumor-suppressive genes. Thus, inhibiting DNA methyltransferase and/or DNA demethylase is a promising therapeutic strategy for many of these cancers. For example, in breast cancer models, the inhibition of DNMT exerts reduced cellular proliferation, migration, and anchorage-independent growth activity and potentiates anti-cancer immunity [41, 42]. Similarly, inhibiting DNMT sensitizes non-small cell lung cancer (NSCLC) to ionizing radiation and a potent targeted therapeutic poly (ADP-ribose) polymerase (PARP) inhibitors [43]. The overexpression of DNMT, particularly DNMT3Ab, in gastric cancer facilitates the epithelial to mesenchymal transition (EMT)-related metastasis and correlates with poor prognosis in gastric cancer patients [44]. Aberrant gene silencing or activation caused by deregulated DNMTs and TETs have also been widely reported in renal, colorectal, brain, pancreatic, bladder, prostate, and other hematological cancers [45]. Among the genes that are implicated in DNA methylation dysregulation include but are not limited to retinoblastoma tumor-suppressor gene (Rb), breast cancer susceptibility gene 1 (BRCA1), cyclin-dependent kinase inhibitor 2A (CDKN2A), and microRNAs [46]. Collectively, the atypical expression of some of these genes leads to genomic instability and uncontrolled cell cycle progression.

Likewise, the PTM of histone tails at gene promoters and on specific residues of non-histone proteins promote different cancer hallmarks. HATs and HDACs regulate acetylation patterns on several proteins and serve as co-activators/repressors of transcription factors implicated in cancer [35, 36]. For instance, in prostate cancer tissues, CBP/p300 transcript levels are significantly high. They potentiate the constitutive activation of androgen receptor signaling in castration-resistant prostate cancer, leading to increased tumor growth [47]. Moreover, the overexpression of the human MYST1, a member of HATs, promotes acetylation of Nrf2 at K588, thereby aiding the tolerance of replication stress in NSCLC [48]. The erasure of acetylation marks is also an important driver of cancer progression. HDACs are typically overexpressed and result in the silencing of key tumor suppressor genes. Particularly, in breast cancer, the use of HDAC inhibitors has shown remarkable potential in preventing hormonal-based therapy resistance through the restoration of epigenetic alterations [49]. A separate review has extensively delineated the role of HDACs in altering

gene expression in cancer through chromatin remodeling and transcription factors regulation [50]. Also, the methylation and demethylation of histone and non-histone substrates have a diverse function in carcinogenesis. For example, the high expression of human telomerase reverse transcriptase (hTERT) observed in many cancers is associated with the heavily trimethylated histone H3K4. H3K4 is a known substrate of SMYD3, a KMT that is commonly overexpressed in cancers [51]. Also, different KMTs such as KMT2A and Dot1-like protein (DOT1L) fuse with proto-oncogenes to promote the progression of hematological malignancies [38]. Another overly expressed KMT in cancer, enhancer of zeste homolog 2 (EZH2) catalyzes the methylation of H3K27 and genes like p16, NF-κB, CDK4, Ras, β-catenin to further different tumors' survival [52]. Similarly, overexpression of KDMs, such as LSD1, LSD2, and KDM5B, cause increased tumor growth and chemoresistance *via* aberrant demethylation of H3K4 in prostate cancer, breast cancer, NSCLC, and hepatocellular carcinoma [53]. A growing number of studies have also documented the widespread role of the known human PRMTs in cancers. The overexpression of PRMTs has been found in breast, prostate, colon, bladder, ovarian, skin, and gastric cancers, including various hematological malignancies [54]. Notably, our group extensively studies PRMT5, and we discovered that its overexpression in pancreatic and colorectal cancer results in increased cell growth, migration, and anchorage-independent growth *via* dimethylation of R30 of NF-κB subunit, p65 [55, 56]. We also revealed that PRMT5 oncogenic role in colorectal cancer potentiates NF-κB signaling through dimethylation of R205 of Y-box binding protein 1 (YBX1) [57]. Taken together, the deregulation of epigenetic enzymes has an entrenched and indisputable role in the etiology and progression of many cancers, making them promising therapeutic targets.

2.2 Epigenetic enzymes implicated in neurodegenerative disorders

Recently, genomic profiling studies and molecular investigations have delineated the impact of epigenetic alterations on neurodegeneration. Neurodegenerative diseases encompass the gradual loss of cognitive and/or motor functions in humans. Examples include but are not limited to Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), Huntington disease (HD), and amyotrophic lateral sclerosis (ALS) [58]. It has been reported that the DNA methylation of AD-associated genes, such as the β-secretase (BACE), amyloid precursor protein (APP), and presenilin 1 (PS1) genes, is dramatically decreased in AD cell models and results in the exacerbation of AD pathology [59]. In a genome-wide study conducted by Huynh and colleagues on MS patients' brains, several differential methylated regions in the DNA were observed. Genes that are critical to oligodendrocyte regulation, such as BCL2L2 and NDRG1, were found to be hypermethylated and showed decreased expression levels [60]. Also, given that HATs like p300/CBP are involved in memory formation, its loss has been shown to lead to different neurological dysfunction, which is characteristic of HD, Rubinstein-Taybi syndrome, and AD [61, 62]. Thus, HDAC inhibitors can be used as a therapeutic strategy to offset the imbalanced role of HATs in the aforementioned neurodegenerative diseases. On the contrary, the downregulation of p300 levels by native α-synuclein (αsyn) exerts neuroprotective function in the brain. Thus, it has been suggested that misfolded αsyn, a major phenotype of PD, may lead to enhanced p300/CBP activity, thereby causing impaired motor function [63]. In ALS, reduced p300/CBP has been found to cause the hypoacetylation of the cyclin D1 gene, a critical gene for cell cycle progression [64]. Similarly, increased methylation marks on histones have been linked with aging, an important risk factor in neurodegeneration [65]. In ataxia-telangiectasia, the loss of A-T mutated (ATM) increases the tri-methylation of H3K27 *via* EZH2 stabilization, thereby affecting neuronal survival [66]. Also, overexpression of an

H3K9 methyltransferase, ERG-associated protein with SET domain (ESET, also known as SET domain bifurcated 1, SETDB1), is shown to be markedly increased in HD patients, and the inhibition of ESET was reported to restore the normal behavioral and neuronal function in HD mice [67]. Moreover, PRMT1 was reported to play a neuroprotective role in ALS *via* asymmetric dimethylation of H4R3, a methylation mark that aids histone acetylation, and consequently, transcription of survival genes [64]. Collectively, this brief overview shows that chromatin modification *via* epigenetic processes is critical to neuronal function.

2.3 Epigenetic enzymes implicated in cardiovascular diseases

Cardiovascular disease, one of the leading causes of mortality globally, is comprised of a group of diverse disorders known to be influenced by genetic, environmental, and epigenetic mechanisms [68]. For example, GWAS on atherosclerotic aorta versus normal aorta showed the differential methylation of DNA is associated with atherosclerotic plaque stability, vascular remodeling, low-density lipoprotein (LDL) signaling, among other biological processes [69]. This suggests the role of altered DNA methylation in the pathogenesis of atherosclerosis. Similarly, case-control investigations on heart failure patients revealed differential methylation of angiogenic genes known to be involved in endothelial cell migration and capillary tube formation [70]. Also, multiple studies have demonstrated that high levels of HDAC and DNA/histone methylation have been linked to the causation of high blood pressure, a known symptom of hypertension [71]. Similarly, the use of HDAC inhibitors attenuates myocardial infarction in *in vivo* studies [72]. A separate study showed that environmental factors, such as particulate matter in air pollution known to cause impaired cardiac function, increase the methylation of Toll-like receptor 2 (TLR2), causing its gene silencing. TLR2 is known to proffer immunity following environmental challenges [73]. Thus, its hypermethylation has been linked to the cardiac dysfunction caused by air pollution. Also, *de novo* mutations have been found in histone-modifying genes in congenital heart disease, including KMT2D, KDM5A, and KDM5B, thereby suggesting their role in the disruption of cardiac development [74]. The overexpression of PRMT6 has been reported to induce cardiac hypertrophy and its associating increase in asymmetric dimethylation of H3R2 promotes the expression of atrial natriuretic peptide (ANP), a hypertrophic marker [75]. This diverse implication of epigenetic enzymes in various cardiac functions suggests its potential as a treatment approach for cardiovascular diseases.

3. Classes of epigenetic enzymes targeted with repurposed market drugs

As discussed in previous sections, epigenetics is pivotal to the etiology and progression of different human diseases. And multiple studies have shown that targeting epigenetic enzymes has a profound effect on attenuating the severity or progression of diseases [59]. Given that the traditional approach to drug discovery is costly and time-inefficient, it is more valuable to reposition readily available market drugs for new disease indications. In this section, we will discuss the major epigenetic enzymes being targeted with repurposed drugs or preclinical compounds and examples of the repurposed drugs with their old and new indications.

3.1 Repurposed drugs for DNMTs

The alteration of DNA methylation is one of the prominent underlying causes of different diseases. Several market drugs have been shown to lessen disease

progression *via* targeting DNMT, suggesting that those drugs could have newer indications. As summarized in **Table 1**, hydralazine is a hypertensive drug that has been repurposed as both DNMT inhibitor and HDAC inhibitors [76]. In combination with another drug, valproate, hydralazine showed a significant increase in progression-free survival in patients with advanced cervical cancer in a randomized phase II clinical trial [95]. Currently, a phase III clinical trial is underway to examine the effect of hydralazine on AD (NCT04842552). It has been suggested that the stability of polyglutamine repeat expansion, an underlying cause of multiple neurodegenerative diseases, can be caused by hypermethylation of the repeat and the use of hydralazine induces demethylation [96]. This may suggest a mechanism through which hydralazine helps ameliorate AD. Another repurposed drug, procaine, a local anesthetic agent, has been reported to be a potent inhibitor of DNMT activity with an anti-tumor effect in gastric cancer [77]. Procaine has also been shown to exert cardioprotective and neuroprotective effects [78]. Other examples of repurposed drugs that target DNMTs include Procainamide, Mithramycin A, Nanaomycin A, and Disulfiram, etc. [79] (**Table 1**).

Table summarizes the different market approved drugs for other indications known to target epigenetic enzymes in newer indications. The listed drugs are either in preclinical or clinical trial phase for their newer indications.

3.2 Repurposed drugs for HDACs

Among the different classes of epigenetic enzymes, HDAC has the highest number of market-approved inhibitors for diseases, especially in cancer [97]. Vorinostat is the first HDAC inhibitor approved by the FDA for cutaneous T-cell lymphoma (CTCL) treatment [98]. Another HDAC inhibitor, Belinostat, has been granted accelerated approval for treatment of relapsed or refractory peripheral T-cell lymphoma (PTCL) [99]. Also, repurposed drugs that target HDAC are on the rise. One category of drugs that targets HDAC is statins. Statins are a class of medications developed to inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase for atherosclerosis treatment [100]. Multiple studies have shown that statins exhibit anti-cancer activity and attenuate diabetic nephropathy *via* the inhibition of HDAC [80, 81]. Similarly, the anti-malaria drug artemisinin was reported to exert anti-cancerous effects on breast cancer cells partly *via* the inhibition of HDAC [82]. Through indirect inhibition of HDACs and other epigenetic modifiers, metformin, a type 2 diabetic medication, has been suggested to have a protective effect on cancer, cognitive impairment, and cardiovascular diseases [83]. Carbamazepine, which is approved for the treatment of psychomotor and grand mal seizures, has been reported to inhibit HDAC 3, 6, and 7 and reduce cancer growth in breast, liver, and colon cancer [101]. Currently, trichostatin A (TSA), an approved antifungal drug with HDAC inhibitory activity, is undergoing a phase I clinical trial for relapsed or refractory hematologic malignancies (NCT03838926) (**Table 1**).

3.3 Repurposed drugs for HATs

The normal levels of gene acetylation can also be restored by HAT inhibitors in diseases. This category of inhibitors is particularly explored as anti-cancer agents, given that inhibiting HATs would only exacerbate cardiovascular and neurodegenerative disease progression. Also, the role of HATs in cancer is context-specific as certain HAT family members can act as oncogenes or tumor suppressors in different tumors. For example, the overexpression of p300/CBP, GCN5, and males absent on the first (MOF) has been shown to sustain cancer hallmarks in glioma, colon, lung cancer, mixed-lineage leukemia (MLL), and acute myeloid leukemia (AML). On the other

Drug	Previous indication	New indication	Epigenetic target	Phase of development	Reference(s)
Hydralazine	Hypertension	Advanced cervical cancer	DNMT	Phase II clinical trial	[76]
		Alzheimer's disease	HDAC	Phase III clinical trial	(NCT04842552)
Procaine	Pain relief	Neurodegenerative and cardiovascular diseases	DNMT	Preclinical	[77, 78]
		Gastric cancer			
Mithramycin	Antibiotic	Lung, esophagus, and other chest cancers	DNMT1	Phase II clinical trial	NCT01624090
Nanaomycin A	Antibiotic	Colon, lung, and bone marrow cancers	DNMT3B	Preclinical	[79]
Procainamide	Ventricular arrhythmias, supraventricular arrhythmias, atrial flutter/fibrillation, and Wolf-Parkinson-White syndrome	Colon cancer	DNMT1	Preclinical	[79]
Statins	Atherosclerosis	Lung, colon, and gastric cancer	HDAC	Preclinical	[80, 81]
		Diabetic neuropathy			
Artemisin	Malaria	Breast cancer	HDAC	Preclinical	[82]
Metformin	Type 2 diabetes	Cancers, neurological disorders, and cardiovascular diseases	HDAC	Preclinical	[83]
Carbamazepine	Psychomotor and grand mal seizures	Breast, liver, and colon cancer	HDAC	Preclinical	[79]
Trichostatin A	Fungal disease	Relapsed or refractory hematologic malignancies	HDAC	Phase I clinical trial	(NCT03838926)
Astemizole	Allergies	Lymphoma	EZH2	Preclinical	[84]
Apomorphine hydrochloride	Parkinson's disease	Alzheimer disease (AD), amyotrophic lateral sclerosis (ALS), Huntington disease (HD), and multiple cancers	EZH2	Preclinical	[85]
Hydroxychloroquine	Malaria	Multiple myeloma	EZH2	Preclinical	[86]
Cloperastine	Cough	Cancers	PRMT5	Preclinical	PCT/US2020/067694 [87, 88]

Drug	Previous indication	New indication	Epigenetic target	Phase of development	Reference(s)
Candersatan	Hypertension	Cancers	PRMT5	Preclinical	PCT/US2020/067694 [87, 88]
Tranylcypromine	Depression	AD	LSD1	Preclinical	[89, 90]
		Cancer			
Phenelzine	Depression	Prostate cancer	LSD1	Phase II clinical trial	[91]
Pargyline	Hypertension	Breast cancer	LSD1	Preclinical	[92]
Nitroxoline	Antibiotic	Leukemia	BRD4	Preclinical	[93]
Azelastine	Hay fever and allergies	Cancer	BRD4	Preclinical	[94]

Table 1.
Examples of repurposed drugs with epigenetic targets in human diseases.

hand, the deletion of p300/(CREB binding protein) associated factor (pCAF) and Tip60 promotes tumorigenesis in certain cancers [35]. Currently, there have been no investigations into the use of market-approved drugs to target HATs in cancer. Natural compounds targeting HATs, such as anacardic acid, plumbagin, garcinol, and lunasin have been reported to have potent anti-cancer properties [79]. Notably, the progression of HAT inhibitors into clinical trials has been challenging due to the resulting false positive hits gotten from HTS [102]. Thus, more effort is needed to find existing market drugs that not only inhibit HATs activity but also attenuate tumor progression.

3.4 Repurposed drugs for HMTs

Given the diverse roles of the two classes of HMTs, KMTs, and PRMTs, in different diseases, there have been increasing efforts towards developing/repurposing drugs that affect HMTs for the treatment of diseases. Under the class of KMT enzymes, EZH2 is one of the highly pursued targets for epigenetic therapy. For example, Astemizole (**Table 1**), an antihistamine drug used to treat allergies, disrupts the proliferation of lymphoma cells *via* inhibition of EZH2 methyltransferase activity [84]. Also, a pilot HTS identified 4 out of 1600 FDA-approved drugs as putative EZH2 inhibitors, with apomorphine hydrochloride being the most potent inhibitor [103]. Apomorphine hydrochloride, under the brand name Kynmobi, is FDA approved for the treatment of PD Off episodes [104]. A separate review has suggested the repurposing of apomorphine hydrochloride in AD, ALS, HD, and multiple cancers considering the mounting evidence that demonstrates its neuroprotective and anti-cancer effects [85]. However, whether this drug exerts its protective properties *via* EZH2 remains to be investigated. The anti-malaria drug, hydroxychloroquine, inhibits EZH2 and has been reported to be effective for the treatment of multiple myeloma (MM) [86]. Furthermore, there are about 10 clinical trial studies investigating PRMT inhibitors for both solid and hematological malignancies on clinicaltrial.gov. Given the lack of investigation on market approved drugs for targeting PRMTs in diseases, our lab has taken considerable efforts to address this important gap. Currently, we have a provisional patent on repurposing the FDA-approved drugs for cough (Cloperastine) and for hypertension (Candersatan) to target PRMT5 in tumors (PCT/US2020/067694) [87, 88].

3.5 Repurposed drugs for KDMs

One of the types of KDMs, LSD1, is a member of the amine oxidase family. Consequently, it shares sequence similarity with monoamine oxidase (MAO), an important enzyme involved in the clearance of neurotransmitters from the brain [105]. As a result, approved monoamine inhibitors, such as the antidepressant tranylcypromine, can also inhibit LSD1 [89]. Notably, tranylcypromine has been reported to suppress amyloid β-induced proinflammatory responses in AD mouse models [89]. Tranylcypromine also reduces tumor growth and metastasis. Hence, its derivatives were developed to optimize the inhibition of LSD1 [90]. One of these derivatives, ORY-1001, is in phase II clinical trial for AML, relapsed, phase I clinical trial for extended-stage disease small cell lung cancer (ED SCLC), and phase I clinical trial for refractory or relapsed acute leukemia (AL). Also, other classes of MAO inhibitors, such as pargyline (anti-hypertensive drug) and phenelzine (antidepressant), inhibit LSD1 with anti-cancer effects in breast and prostate cancer, respectively [91, 92]. On the other hand, the second class of KDM, the JmjC KDM, is yet to be investigated for market drug repurposing. Nonetheless, a couple of pharmaceutical companies are taking strides to develop inhibitors against this class of KDM in hematological and solid cancers [32].

3.6 Repurposed drugs for BETs

BET protein family, including BRD2, BRD3, BRD4, and BRDT, are readers of acetylated K residues on histones and non-histone proteins. BET inhibition is effective against kidney diseases, tumor development, cardiovascular disease, and other inflammatory diseases [106]. Some drugs have been repurposed to target BET proteins in diseases. For example, nitroxoline (**Table 1**) is an FDA-approved antibiotic and also a potent inhibitor of most BET family members. A study reported that nitroxoline significantly reduced the proliferation of leukemia cells *via* induction of apoptosis and cell cycle arrest. The anti-cancer action of nitroxoline is partly through BET inhibition and its downstream targets [93]. Another class of BET inhibitors, for example, molibresib, is a derivative of benzodiazepines, a psychoactive class of drugs used to treat neurological-related conditions. Molibresib is currently in phase I clinical trial for the treatment of multiple cancers [107]. Additionally, azelastine, an antihistamine used to treat hay fever and allergies, was ranked as one of the top drugs for having the best binding affinity to BRD4 [94]. Collectively, the aforementioned approved and putative repurposed drugs could serve as an effective BET inhibition-based therapy in different diseases.

4. Recent advances in drug repurposing for epigenetic-based therapy

Repurposing drugs for epigenetic-based therapy is a newly emerging field with significant potential for the development of drugs for diseases with high incidences, such as cancer and cardiovascular diseases. Notably, epigenetic enzymes play a critical role in the molecular pathology of the diseases discussed in this chapter. Thus, it is important to increase the development of drugs targeting epigenetic enzymes in a timely and cost-efficient manner. Due to the increased development of HTS methods, availability of comprehensive omics data, and advances in computational tools, the use of drug repurposing as a therapeutic strategy is highly promising. Through literature database search, researchers can often extrapolate the potential efficacy of a market-approved drug in a new indication based on the drug's molecular effect and cellular impact in an older indication. Such information opens a window of opportunity to examine the use of market-approved drugs in a new indication. For example, researchers observed that artemisinin, an approved malaria drug derived from the wormwood plant, forms free radicals with iron. Considering that increased iron levels are a well-established risk factor for breast cancer development, an investigation was launched into the anti-cancer effects of artemisinin [82]. Moreover, recent studies in epigenetic-based therapy have also adopted molecular docking tools to identify valuable drug candidates that can be repurposed for new indications [94, 108]. The study of the target structure and ligand interaction significantly scales down the evaluation of drugs that are unlikely to bind to the epigenetic targets that fuel a disease progression. This approach also leverages structural similarities of a market-approved drug's target to discover potential newer indications. Notably, our group developed an AlphaLISA-based high-throughput screen (HTS) that aided the identification of promising market-approved drug candidates which targets PRMT5. This unique HTS method allowed us to preclinically investigate the efficacy of candesartan and cloperastine, a hypertensive and cold medicine, respectively, in several solid cancers [87, 88]. As with other drug discovery approaches, the exciting advances in targeting epigenetic enzymes with market-approved drugs can be improved with additional extensive research on various aspects of the drug's molecular mechanisms. In some cases, although a

repurposed drug is known to have an epigenetic effect, its primary molecular target is not always clear. This gap creates an avenue for the possibility of off-target effects that may be adverse in newer indications. Similarly, the challenges with false-positive results in HTS can be surmounted by incorporating the dose-response factor as a critical variable for understanding a market-approved drugs' efficacy against an epigenetic target. Also, considering that the function of a market-approved drug can be context-dependent, it is critical to pursue new indications known to be highly driven by an epigenetic target of interest. Collectively, addressing the gaps in molecular mechanisms that drive disease pathology and improving existing screening methods will significantly advance the field of epigenetic-based therapy using market-approved drugs.

5. Future perspectives

Given that the *de novo* drug discovery approach for epigenetic targets is time-consuming, costly, and has a high failure rate in clinical trials, researchers may consider increasing their efforts into repurposing drugs with known epigenetic effects for newer disease indications. One of the merits of drug repurposing is that it alleviate patients' treatment costs and provide hope to those with rare conditions. Recognizing this approach as a great benefit to patients, governmental agencies and philanthropic organizations should increase the establishment of funding programs for drug repurposing endeavors [109]. More importantly, the paradigm for drug discovery is moving from a single target to a multitarget approach and drug repurposing is a suitable strategy to meet this evolving paradigm in pharmacology [110]. Thus, considering the slow pace and millions to billions of dollars spent on bringing a single drug to market, it is worthwhile to steer efforts and resources towards drug repurposing for epigenetic-based therapy in human diseases.

6. Executive summary

- Drug repurposing is a creative approach to drug discovery that comprises finding new indications for approved drugs in the market or drugs that have been recalled/inefficacious in a previous indication.
- The advent of computer-aided drug discovery and the HTS methods have significantly accelerated the drug repurposing process.
- The extensive role of molecular targets such as DNMTs, HDACs, HMTs, KDMs, and BETs in several human diseases necessitates the development of drugs to alleviate the progression of diseases driven by the aforementioned epigenetic enzymes.
- Thus, epidrugs (drugs that target epigenetic marks) have been widely incorporated in the management of diseases such as cancer, cardiovascular diseases, kidney disease, and neurological disorders.
- Recent advances in the drug repurposing approach increased the use of market-approved drugs to target epigenetic enzyme-driven diseases.
- Currently, several market-approved drugs have shown significant pre-clinical efficacy in diseases and/or are undergoing clinical trials for new indications.

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Therapeutic Inhibitors: Natural Product Options through Computer-Aided Drug Design

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Abstract

Drug repurposing involves reusing an active pharmaceutical ingredient that is already in the market and drugs that were unsuccessful in their clinical phases of development for a new indication. It has numerous benefits in drug development. Therapeutic inhibitors are agents that could be of synthetic or natural source with the ability to trigger the down-regulation of an enzyme or protein, thereby inducing therapeutic effect(s). Researchers have embraced synthetic methods in searching for therapeutic molecules through structural activity relationships and other means in the past and recent times. Despite these synthetic drugs, the morbidity and mortality rate of ailment and disease affecting humanity remains overwhelming. Research has shown that solutions to these challenges can be attempted through drug repurposing. In the past, natural products in raw forms have been utilized in traditional, complementary medicine to manage and treat diseases and illnesses, as there are molecules in use today as drugs, which originated from plants and other natural sources. Studies on natural products have led to diverse natural product databases that can serve as a source of repurposing agents. There are also databases for protein and enzymes of human origin, which have an enormous role in the in-silico drug repurposing approach.

Keywords: repurposing, therapeutics, inhibitors, *in-silico*, protein database, natural products

1. Introduction

Repurposing of a drug is the process of reutilizing already utilized drugs for other treatment purposes. It is the use of a known drug for treating conditions other than their primary use [1]. Drug repurposing encourages disease-related drug development in a much cheaper, faster, and more accessible way for patients [2]. The drug studied for repurposing is the shelved drugs, drugs in use, discontinued drugs, and experimental drugs that either could not make it to the late phases of clinical trials or have failed in the market. Because the efficacy, safety, and toxicity of these drugs have already been established, the preliminary phases of the clinical trials can be omitted, minimizing the cost and length of the clinical

trials. It takes about 15 years to deliver a new drug to the market [3]. The objective of drug repurposing is to identify new biological targets and different therapeutic uses of previously approved and/or investigational drugs, including drugs that did not meet primary therapeutic expectations. As such, a number of pre-clinical development and optimization issues, including negative toxicological profiles, can be avoided or at least minimized. Although most successful experiments in reallocating drugs are derived from coincidence, current research efforts focus on predicting opportunities for reallocating on rational grounds [4]. Interestingly, while most drug reallocation campaigns rely on chemical-based compounds, natural products can offer important opportunities. Natural products are characterized by unique and favorable properties, considerable structural diversity, and a large number of pharmacological activities [5]. Therefore, these are chemical entities preferred for the (re)discovery of medicines. Strategies that may bring to light new therapeutic uses that may not be related to their original biological space [6].

In the drug repurposing process, there are three important processes that are involved. They include (i) identification of the targets of interest for a new indication, (ii) assessment of mode of action intricate in drug or ailment of study, and (iii) establishment of the drug the usefulness in the second and third phases of a clinical trial. Of all the stages, finding a principal candidate is one of the most important. This is the stage where the most advanced and efficient techniques are required to be involved in generating new hypotheses in the reallocation of drugs. Drugs can be repurposed in multiple ways, which may be either experimentally, clinical-based, or computationally. The computational approach is an "*in-silico*" repurposing of drugs, which is divided into two sub-categories: centered drugs or diseases. Under the drug-based approach, we find new indications for existing drugs, while under the disease-based approach, we try new drugs for an existing disease (**Figure 1**) [7].

Recently, natural products have seen a revival of awareness in drug discovery, with a different approach. Newer and evolving technologies, such as computational screening, proteomics, metabolomics and big data analysis, have come to the fore to drive and speed up the "repurposing" of natural compounds and, more generally speaking, of nature-inspired compounds [8].

Even though a large number of natural product formulations are available as extracts, the phytocompounds components of these extracts can be utilized in drug repurposing, with the utilization of Computer-Aided Drug Design, after isolation, purification, and structural elucidation.

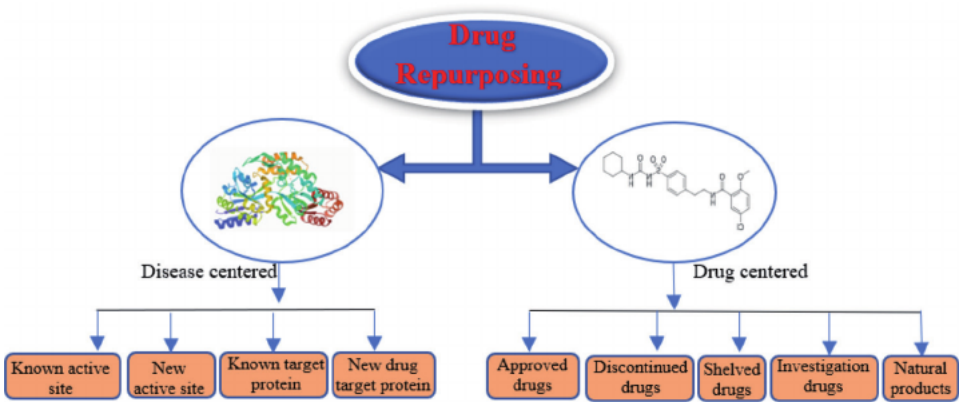

Figure 1.
Drug repurposing approaches.

Approaches for Speeding Up Drug Repurposing

- *In-silico* models—*In-silico* or bioinformatic models help to identify complex relationships between drugs, targets, and diseases necessary for reuse [9].

- Target Linkage—The use of high-throughput assessment technological skills to identify multipharmacological molecules that affect numerous targets can remedy multifactorial ailments such as cancer and diseases of neurodegeneration [10].

- Artificial Intelligence (AI)—AI makes records more accessible. Broad literature mining to identify possible drug interactions, adversarial effects, mode of actions, regulations of a gene can help accelerate the development of medicines [11]. The side effect of the medicine may be utilized to treat another condition. If the medications have the same adverse reactions, then they can work on the same disease [2].

Therapeutic inhibitors are agents, compounds that could be of synthetic or natural source, with the ability to trigger the down-regulation or block the expression or overexpression of an enzyme or protein, or block protein-protein interactions or block the addition of phosphates to other molecules, thereby inducing therapeutic effect(s). Therapeutic inhibitors perform their functions either directly or indirectly by affecting the catalytic properties of the active site. Inhibitors can be extraneous to the cell or normal constituents of it. Inhibitors which are a normal component of a cell, can represent a significant component of the regulation of cell metabolism. Many toxins and also pharmacologically active agents (both illegal drugs and prescription and over-the-counter medicines) act by inhibiting specific enzyme-catalyzed processes [12], which can be targeted *in-silico* using computer-aided drug design in the process of new therapeutic inhibitor development from natural products. There are thousands of natural products existing in natural product databases that can be utilized for this purpose. The protein and enzyme target are also readily available in protein databases in formats needed for computer simulation studies [13].

2. Classifications of therapeutic inhibitors

Based on the current mechanisms of action of already existing drugs, therapeutic inhibitors can be classified into

1. Enzyme inhibitors

2. Protease inhibitors

3. Kinase inhibitors

4. Protein synthesis inhibitors

5. Protein-protein interactions inhibitors (**Figure 2**).

2.1 Enzyme inhibitors

Enzyme inhibitors are compounds that interact with enzymes (either temporarily or permanently) in some way and minimize the rate of an enzyme-catalyzed

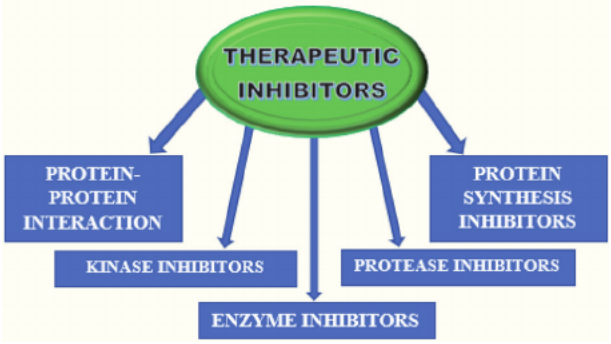

Figure 2.
Therapeutic inhibitors.

reaction or stop enzymes from working in a normal manner [13]. In therapeutic, enzyme inhibitors bind to enzymes and lower their activity [14] and achieve a therapeutic benefit. Some molecules are used as drugs today because of their ability to cause correction of metabolic imbalance, the correction which is due to the effectiveness of the molecules in causing blockage of enzyme activity. Therefore, the search and discovery of molecules with inhibitory enzyme ability is an active area of research in biochemistry and pharmacology [14]. It is noteworthy to state that not all molecules that bind to enzymes are inhibitors; some could be enzyme activators; in this case, the molecules bind to enzymes and elevate their enzymatic activity [15], which can also cause therapeutic benefit. The binding of inhibitors to enzymes can either be reversible or irreversible.

A molecule is a reversible inhibitor if it binds non-covalently to the enzyme's active site to produce an inhibition. The binding could be direct with the enzyme, the enzyme-substrate complex, or both [15].

A reversible inhibitor is described as one that, once removed from the enzyme, the enzyme returns to its normal function pre-inhibition. It exerts no permanent effects on the enzyme and does not change the shape of the active site of the enzyme [16]. There are different types of reversible inhibition. They include competitive, non-competitive and uncompetitive types, although a mixed type sometimes arises [15].

The underlying principle of competitive inhibition is that, at a single active or binding site of a drug-metabolizing enzyme, there is the mutually exclusive binding of either the substrate or the inhibitor [17]. Competitive enzyme inhibitors possess a comparable shape to that of the substrate molecule. These two drugs compete for binding to a single active site of an enzyme. Substrates are compounds or molecules upon which enzymes act. The interaction of a substrate and an enzyme occurs at the active site of the enzyme or in a binding site that can, in turn, alter the active site. This brings about competition for binding/active sites between a substrate and an inhibitor.

The second type of reversible inhibition, non-competitive reversible inhibition, utilizes inhibitors that do not have similarity with the substrate and so do not bind to the active site but rather to a separate site on the enzyme. The outcome of an interaction of a non-competitive inhibitor with an enzyme appreciably differs from an interaction with a competitive inhibitor due to the non-existence of antagonism. In the case of an antagonistic inhibition, the inhibitory effect could be minimized and subsequently overcome with escalating concentrations of substrate. With non-competitive inhibition, growing the quantity of substrate does not affect the percentage of an enzyme that is active. Indeed, in non-competitive inhibition, the

percentage of enzyme inhibited remains the same through all ranges of a substrate. The implication of this is that non-competitive inhibition will efficiently diminish the concentration of enzyme by equal, fixed concentration in a typical experiment at every substrate concentration used [18].

The third type of reversible inhibition, uncompetitive reversible inhibition, utilizes inhibitors that bind to the already formed enzyme-substrate complex and not to the free enzyme. In this type of reversible inhibition, the interaction of the substrate with an enzyme could trigger a conformational modification that leads to the revelation of an inhibitor binding site on the enzyme, or the inhibitor could bind and interact directly to the enzyme-bound substrate. The underlining outcome in this type of reversible inhibition is that it does not compete with the substrate for the same active site in either case and so the increasing concentration of substrate cannot overcome the effect of the inhibitor [19].

As opposed to reversible inhibition, there is irreversible inhibition. In irreversible inhibition, the inhibitor no longer separates from the enzyme after binding and interaction and the enzyme reaction is reduced. The reduction rate is dependent on the enzyme and inhibitor concentrations only and independent of the concentration of the substrate. This implies one inhibitor molecule can ideally minimize to zero the activity of one enzyme molecule [20]. Irreversible inhibition could be of two forms. The first occurs when an inhibitor is strongly bound and complex with an enzyme and fail to dissociate under physiological conditions from the enzyme. There are two types of irreversible inhibitors. The first type is so strongly complexed to the enzyme that it fails to dissociate from the enzyme under physiological conditions but can be dissociated through the method of dialysis or by chromatographic techniques [20]. The second type of irreversible inhibition is one in which the inhibitor forms a covalent bond with the enzyme; in a situation whereby the formation of the covalent bond terminates the conversion of substrate to product, then the enzyme has been irreversibly terminated. The irreversible inhibitors that function through the formation of covalent bonds are of two main types. The first type involves the reaction of an inhibitor with an essential functional group by a bimolecular process on the enzyme [21]. The biomolecular process is a reaction that involves the combination of two molecular entities. In the second type of irreversible inhibition that occurs through the formation of a covalent bond, the inhibitor which bears a leaving group forms a reversible complex with an enzyme. As this occurs, the presence of a nucleophilic group on the enzyme of the leaving group, juxtaposed within the reversible enzyme-inhibitor complex formed on the enzyme of the leaving group, could lead to a rapid neighboring group reaction within the complex in which a covalent bond is formed. Such formation of a covalent bond can be highly specific since properly positioned neighboring groups can react more rapidly than the identical bimolecular reaction [21]. A leaving group is an atom or group of atoms that dissociates from the rest of the molecule, taking with it the electron pair, which was previously the bond between the leaving group and the rest of the molecule.

2.2 Kinase inhibitors

Kinase is a type of enzyme that acts to add phosphates to other molecules, such as sugars or proteins. The addition of phosphate may cause other molecules in a cell or system to become either active, overactive, or inactive. Kinases facilitate the transmission of a phosphate moiety from a high-energy molecule to its substrate molecule. Kinases are widely utilized to convey signs and control multifaceted procedures in cells. Phosphorylation of compounds can boost or impede their effectiveness and regulate their capability to interrelate with other compounds.

The presence and absence of phosphoryl groups offer the cell a means of control because various kinases can react to diverse situations or signals. There are 518 kinases encoded in the human genome are 518 kinases. These kinases are known to phosphorylate about one-third of the proteome [22, 23]. Nearly all signal transduction route occurs through a phosphotransfer process. This indicates that kinases offer several nodes for therapeutic mediation in numerous abnormally controlled biological processes [24]. Kinase function deregulation has been shown to perform an essential role in cancer immunological, inflammatory, degenerative, metabolic, cardiovascular and infectious diseases [25, 26].

Kinases are of three main categories depending on the substrate type of kinase: protein kinase, lipid kinase, carbohydrate kinase. Protein and lipid kinases represent one of the most important target classes for treating human disorders after G-protein-coupled receptors (GPCRs) and proteases. As a matter of fact, one-third of the protein targets currently undergoing investigation by pharmaceutical companies consist of protein or lipid kinases [27].

Kinase inhibitors are molecules with the ability to alter the activities of kinases. The recognized druggability and the therapeutic safety profile of standard kinase inhibitors make kinases attractive targets for drug development. Nevertheless, there are many kinases yet to be studied effectively; this shows that the discovery of kinase inhibitors is still the majority of kinases that have been historically understudied, indicating that the field of kinase inhibitor discovery is still not fully harnessed [28–30]. There are some significant challenges in drug discovery as regard kinase inhibitors. These challenges are obstacles to the full potential of kinases as drug targets. The challenges include validating novel kinase targets, utilization of kinase inhibitors in non-oncology therapeutic areas, overcoming drug resistance, obtaining target selectivity to minimize off-target-mediated toxicity and to develop effective compound screening and profiling technologies [31]. Nevertheless, some progress has been made in towards overcoming these challenges, and also research in the field of kinase inhibitors have Over the course of the past 5 years, immense progress has been made towards these goals, and also studies the field of kinase inhibitor discovery is expanding rapidly in oncology and into different disease areas, including autoimmune and inflammatory disease as well as degenerative disorders.

The estimated current spending in research and development by pharmaceutical companies towards the development of new kinase inhibitors is about 30%. In all these, one of the most important classes of drugs targeted by pharmaceutical industrial researchers is protein kinases. To date, 89 drugs targeting protein kinases have clinically received approval. It is estimated that the current global market for kinase therapies is about US$20 billion per annum, projection to rise distinctly. Over 100 active small-molecule kinase inhibitors are currently in an advanced stage of clinical development, and many more are expected to be approved in the years ahead [32].

2.3 Protease inhibitors

Proteases, which are also known as proteinases or proteolytic enzymes, are a large class of enzymes that catalyzes the hydrolysis of peptide bonds in proteins and polypeptides. Proteases control the fortune, localization, and numerous protein actions. Proteases are important aspects in the well-being and viability of cells, participating in several procedures, such as replication, transcription, cell multiplication, differentiation, extracellular matrix remodeling, and processing of hormones and biologically active peptides. Proteases are greatly controlled (e.g. transcriptionally, post-translationally, stimulated, inhibited, and classified) [33]. Protease action has been found to play a role in the pathogenesis of vascular diseases, including

atherosclerosis, thrombosis, and aneurysm. Broad diversity of proteases representing various proteolytic groups and their corresponding inhibitors are involved. These proteases play a role(s) vascular ailment through a sequence of overlapping pathways that upset the overall inflammatory status and structural integrity of the vessel wall. By triggering PARs (protease-activated receptors), these enzymes cause inflammatory signaling, cytokine production, and inflammatory cell recruitment. Furthermore, proteases can destroy components of the extracellular matrix (ECM), elastic lamina, and fibrous cap in the atheroma. The fundamental paradigm is that excessive proteolytic action is an important contributor to the start and progression of vascular disease. Recent approaches to the treatment of vascular pathologies have attempted to modulate protease activity in an effort to reduce inflammation and preserve the structural integrity of the vessel wall [34]. Proteases can be divided into six broad classes based on proteolytic mechanism: serine proteases, threonine proteases, cysteine proteases, aspartic proteases, metalloproteases, and glutamic acid proteases.

Protease inhibitors are synthetic drugs that prevent the activity of HIV-1 protease, an enzyme that cleaves two precursor proteins into smaller fragments. These fragments are essential for viral growth, infectivity, and replication. It is important to mention that proteases are not limited to HIV. Protease inhibitors interact with protease at the active site, thereby thwarting the growth and development of the freshly formed virions; this makes them stay non-infectious. Protease inhibitors are utilized in taking care of individuals with human immunodeficiency virus (HIV infection) and acquired immune deficiency syndrome (AIDS) [35]. Also, protease inhibitors are useful medically as angiotensin-converting enzyme inhibitors for blood pressure, proteasome inhibitors for myeloma, dipeptidyl peptidase IV inhibitors for type II diabetes [33]. Currently, there are many studies in progress targeting SAR-COV-2 main protease (Mpro) [36–41]. Mpro, also termed 3CL protease, is a 33.8 kDa cysteine protease that mediates the maturation of functional polypeptides involved in the assembly of replication-transcription machinery [42]. Due to the significant role of this main protease, it is considered a promising drug target, as it is dissimilar to human proteases.

2.4 Protein synthesis inhibitors

The process of making a protein molecule using DNA, RNA, and various enzymes by cells is termed protein synthesis. In biological systems, it takes place inside the cell and involves amino acid synthesis, transcription, translation, and post-translational events. It takes place in the cytoplasm of prokaryotes, while in eukaryotes, it takes place usually in the nucleus and aids the generation of a transcript (mRNA) of the coding region of the DNA. The transcript departs the nucleus and gets to the ribosomes, where translation into a protein molecule takes place with a specific sequence of amino acids [43].

A protein synthesis inhibitor is a molecule with the ability to terminate or reduce the growth rate of cells by interrupting the progressions that directly leads to the production of new proteins [44]. Even though a wide description of this definition could be utilized in closely describing any compound depending on the amount present, in reality, it classically denotes compounds that exert their molecular effect level on translational machinery. Protein synthesis inhibitors are another major group of therapeutically useful antibacterials, such as erythromycin, tetracycline, chloramphenicol, and aminoglycosides. They specifically interact with the 70S bacterial ribosome and spare the 80S eukaryotic ribosome particle. Macrolide, lincosamide, and streptogramins (MLS) antibiotics represent three classes of structurally diverse protein biosynthesis inhibitors used clinically [45]. Generally, protein

synthesis inhibitors work at different stages of bacterial mRNA translation into proteins, like initiation, elongation (including aminoacyl tRNA entry, proofreading, peptidyl transfer, and bacterial translocation) and termination.

2.5 Protein-protein interactions inhibitors

The protein-protein interaction (PPI) can be described as a substantial network linking a protein and its partner(s) [46–48]. These networks may exhibit a variety of heterogeneities and complexities in large molecular structures, leading to the formation of protein dimers, multi-constituent complexes, or lengthy chains [49]. The contact between subunits of protein can be transitory or constant, similar or dissimilar, and precise or imprecise [48, 50, 51]. There are closely 650,000 protein-protein interactions in humans, and this figure keeps on increasing as additional interaction networks are being discovered [48, 52]. Protein-protein interactions (PPIs) play pivotal roles in biological processes [53]. Mutations or compromised regulation of PPIs affect cellular networks and have a role to play in the development of diseases. The discovery and development of new PPI inhibitors with the intention to control abnormal pathways have therefore aroused substantial interest from the pharmaceutical industry [54]. Almost half of the dry mass of a cell is composed of proteins, and disorder in PPIs often causes diseases, including cancer [55, 56]. Hence, research and studies on PPI play a vital role in advancing our understanding of molecular biology and human diseases, as well as for developing new therapeutic agents in drug discovery [51, 57, 58].

Generally, protein-protein interactions were used to being seen as a non-druggable target. This standing is likely due to the lack of or limited knowledge on high-throughput assessment assays, as well as the consideration that most protein-protein interactions are held to by big, chemically noncomplex surfaces with a deficiency of easily druggable pockets [59]. While such tough protein-protein interaction targets indisputably exist, it is now understood that many protein-protein interactions use minimal interfaces for their interaction, regularly consisting of an unstructured peptide bound to a distinct groove [54]. Additionally, mutagenesis analyses of numerous PPIs have shown that surfaces causing the affinity of a given PPI are not steadily spread across the whole interface. Rather, there tends to be a "hot spot" or a small number of important residues that anchor two proteins together [60]. This implies that a putative inhibitor would not need to dislodge the entirety of a given PPI but rather only occupy the hot spot, a more tractable problem.

Currently, researches in the area of SARS-COV-2 also include inhibition of Spike-ACE2 interaction, which is a protein-protein interaction [61, 62].

3. Natural products option

Plants as a source of medicine Nature, as old as mans' existence, have been a provider of medicines and agents used for the development of medicine. There are other natural sources of medicinal products like marine, but the most prevalent source is a plant [63]. An enormous number of the medications in use today was obtained from a plant. Some medications in use also were developed from a compound originally gotten from a plant. The development of most of these medicines gotten from a plant started from the study of the utilization of the plant in traditional medicinal practice, which gave an insight into the type of pharmacological property or likely pharmacological effect for which the molecules from plants could be developed for.

The system of traditional medicinal practice keeps on being a very indispensable target in the world's healthcare system because of the dependence of the 80% of the world population on traditional medicinal practice system for their elementary healthcare needs, according to World Health Organization. The remaining 20% of the population who are residents of developed countries also use plant products for healthcare needs or substances developed from plant products [64]. Research has it that between 1959 and 1980 in the United States that about 25% of the dispensed prescription drugs from community pharmacies were products of plant extracts or contained active ingredients obtained from higher plants [65].

Currently in use as medications are at least 119 chemical entities obtained from 90 different plant species. Of all these 119 drug entities, 74% were obtained from plants through direct isolation of active substances from plants that are already in use in traditional medicinal practice systems [66]. It is on record that in all sales made by the leading pharmaceutical industries in the year 1991, most of the sales were made on products derived from natural sources or containing a substance or substances that are natural product-based [67]. It is also on record that in 1993, a total of 57% of the top 150 brand-name products that were prescribed had at least one major active compound from a natural source or derived from a natural source or patterned after substances reflecting biological diversity [68].

Many researchers have taken an interest in discussing and accessing different medicinal plants as a reservoir for new therapeutic agents [63], and some others have persuasively converged their research on the use of specific chemical classes like flavonoids, alkaloids, glycosides, etc. in drug discovery. Recent research has continued to demonstrate and validate the ethnomedicinal drug discovery approach to the initial discovery approaches of pharmaceuticals [69]. Still, some other researchers have estimated that out of about 375 total compounds of pharmaceutical importance in the rain forests, only about one-eight have been explored. Assessing and observing the roles these natural product base medications have played in humanity, there are possibilities that more efficient ones are still in the forest unexplored [70].

This forms the basis for the need for more exploration and research on traditional medicinal plants for the emerging healthcare challenges of humans. Researchers in the field of medicinal plants are no longer only interested in testing plant extracts for pharmacological activities but are also undertaking the isolation of molecules from plant extracts and identifying these molecules. Some has gone further to establish the pharmacological effect of these isolated molecules. For example, in previous research on Vernonia amygdalina, we were able to establish the antidiabetic and antihelminthic effectiveness of methanolic extract Vernonia amygdalina [71, 72], and we went further to isolate six pure molecules from the methanolic extract [73] and then tested the isolated molecules for the antidiabetic and antihelminthic property. From the study, we were able to identify the molecules responsible for the antidiabetic and antihelminthic effects observed in the extract [74].

Likewise, there are thousands of isolated molecules from plants yet to be studied for any pharmacological activity. These molecules form plants are usually deposited in natural product databases from where their structures can be downloaded for studies using Computer-Aided Drug Design. Some of them are also available for purchase for in-vitro and in-vivo studies. Some of the natural product databases include;

- ColleCtion of Open NatUral producTs (COCONUT) [75], containing 406,747 phytocompounds

- African Natural Products Database (ANPDB) [76, 77], containing 6515 phytocompounds

- Comprehensive Marine Natural Products Database (CMNPD) [78], containing 32,000 compounds

These isolated and identified natural products can also serve as a primary source for new molecule development through *in-silico* structural modification and synthesis. The existence of these natural products and these databases has provided a vast background for targeted natural product drug design and development. To be able to utilize these natural compounds in receptor/protein/enzyme targeted drug design and development *in-silico*, the receptor/protein/enzyme need to be available in a portable format that will enable its utilization *in-silico*, which is provided in protein databases.

4. Protein databases

A protein database is a body of data derived from physical, chemical and biological information about the sequence, domain structure, function, three-dimensional structure, and protein-protein interactions. Together, protein databases can serve as a database of protein sequences. Therefore, it is significant to utilize suitable protein databases that can analyze and store data relating to protein science and also expedite the utilization of analytical software accessible to the scientific community. Protein databases can be broadly grouped into two types. The first is a universal type, a set of proteins found in all identified biological species. The second kind of protein database is a specialized database that deals with proteins belonging to a specific group or family of certain species. In addition, each protein database can be further categorized according to the type of information required [79].

4.1 Categories of a protein database

Since protein datasets are being developed from different experimental groups, it would be necessary to provide suitable databases to meet their needs. Presently there are several types of protein databases accessible to the public, which can be further classified into more specialized categories based on the type of information sought [79].

4.1.1 Protein sequence database

Protein sequences consist of 20 different amino acids; this sequence is known as the primary structure of a protein. This type of protein database, which collects amino acid sequences of proteins and related information, is termed a protein sequence database. Examples of this type of database include; Swiss-Prot [80], TrEMBL [80], PIR [81], DDBJ [82], etc.

4.1.2 Protein structure databases

Protein structure regulates function, given that the specificity of active sites and binding sites hinges on the exact three-dimensional conformation. Protein structure databases contain information related to three-dimensional protein structure and secondary structure obtained from analyses by X-ray crystallography, electron microscopy and NMR. Examples include Protein Data Bank (PDB) [83], etc.

4.1.3 Protein-protein interaction databases

A protein-protein interaction database is developed on the basis of protein-protein interaction information gotten from yeast two-hybrid, co-purification, affinity column chromatography, in vitro binding and IP/coIP (protein immunoprecipitation (IP)/ co-immunoprecipitation (Co-IP) methods. Examples include; BIND (biomolecular interaction network database) [84], DIP (database of interacting proteins) [85], MINT (molecular interactions database) [86], etc.

4.1.4 Protein pattern and profile databases

Motifs can be identified in protein, DNA, and RNA sequences, but the most familiar use of motif-based analysis is the identification of sequence motifs conforming to structural or functional features in proteins. One of the essential instruments for sequence analysis is the utilization of protein sequences or profiles to establish protein function [87, 88]. Example, Interpro [89], etc.

4.1.5 2-D PAGE databases

These 2-D PAGE databases comprise gel image data acquired by examining the 2-DE and documented data on gel spots about molecular mass (M.W.), isoelectric point (pI), a status report on the identified location, and cross-reference links [90].

4.1.6 Metabolic pathway databases

Metabolic databases offer descriptive data on enzymes, biochemical reactions and metabolic pathways. Examples are BioCyc [91], MetaCyc [92], etc.

4.1.7 Signaling pathway databases

This signaling pathway database is to inspire complementary investigation in individual laboratories and to enable access to essential information on biological signaling pathways. This database can be classified into the following areas, depending on the format, for it contains both graph and tree-type data structures.

Examples are TRANSPATH [93], etc.

With these receptor/protein/enzyme databases and natural product databases, more *in-silico* research aiming towards the discovery and development of more therapeutic inhibitors from natural products can be initiated. At the present time, *in-silico* approaches have become an essential aspect of the drug discovery procedure. The use of *in-silico*/ computational approaches to discover, develop, and analyze drugs and similar biologically active molecules is referred to as Computer-Aided Drug Design.

5. Computer-aided drug design/repurposing

Computer-aided drug design, which commenced in about the early 1970s, is a process where new drug molecules are designed/identified, redesigned or repurposed to bind with a biological target of known or predictable 3D structure and express substantial affinity/specificity [94]. The core purpose of drug design methods is to utilize the receptor/ligand tertiary structures for accelerating the drug discovery process and also repurposing or enhancing the inhibition properties of a ligand, which could act as a therapeutic inhibitor. In performing computer-aided

drug design, two approaches can be implemented. The first is structure-based (target-based), while the second is ligand-based (analogue-based) [95].

Methods by which the 3D structure of a protein can be generated include X-ray crystallography, NMR, electron microscopy, or prediction based on homology in silico. Once the 3D structure has been resolved, the protein's binding site or active site is identified. Structural-based drug design methods recognize/design an inhibitor having functional properties complementary to the protein binding site. These include molecular docking and the design of de novo molecules. Molecular docking techniques assess a molecule's most viable binding geometries at the binding site of a target protein in the 3D space. These binding geometries are termed binding poses, which include both configurations, which are the molecule's position in the target or the receptor-binding site and conformational sampling. These binding geometries are recorded using molecular mechanics and calibrated according to the intensity of the interaction with the receptor. This process can be performed on large high-speed databases (virtual screening), allowing rapid molecular screening to recognize the right inhibitors. De novo design approaches form ligands that have not been synthesized before. In this approach, the functional groups responsible for interactions with the target receptor are positioned in the additional 3D space of the protein binding site and are linked to the binding scaffolding. This technique assumes that only the functional groups of a molecule are responsible for their activity and not the scaffold [96].

Ligand-based drug design approaches like quantitative structure-activity relationship (QSAR) and pharmacophore modeling have established their effectiveness in designing/envisaging the action of new molecules and in searching chemical databases to detect novel lead scaffolds in the absence of target receptor 3D structure [97–99]. QSAR and quantitative structure-property relationship approach developed a mathematical model for biological activity employing numerous structural and functional properties [100–102]. This activity (dependent quantity) and property (independent quantity) model can be used to contemplate the activity of novel molecules as inhibitors without knowing the structure of the 3D receptor. These relations can be obtained using statistical measurements such as regression approach, neural networks, main component analysis (PCA), partial least squares (PLS).

These days *in-silico* drug repurposing is attracting global awareness as a result of the accessibility of a huge amount of data on protein structures, pharmacophores, disease data, clinical investigations, or gene expression profiles of medicines. As well, increased public social networking technologies and computational access to genetic information have greatly helped computational approaches predict new indications. As a result, most pharmaceutical companies use bioinformatics or modern computing resources to reposition drugs from various chemical spaces. The ultimate desire of each pharmaceutical company is to be able to put medication into the market with increased speed and at the same time lower the cost of design and development. The powerful in-silico technology can provide these benefits. With the increase of drug-related data available, new computational approaches with improved recall and precision for targeted profiling of small compounds have been developed. These approaches enhance the repurposing procedure by including chemoinformatics, bioinformatics, network biology, systems biology or genomic information to uncover unidentified targets and mechanisms for approved drugs with accelerated timeframes.

6. New therapeutic inhibitors and natural products

In utilizing existing drugs in drug repurposing and natural molecules in the discovery and development of new therapeutic inhibitors, all we have discussed above:

classification of therapeutic inhibitors, protein database, natural product database and Computer-Aided Drug design, have essential roles to play.

Before one can aim at targeted drug design, there must be a disease of interest at heart. Then, understanding the pathophysiology and pathogenesis of the disease. A good understanding of these processes to identify the protein and enzymes involved in the pathophysiology and pathogenesis and also the role(s) each of these proteins and enzymes plays. Apart from the role the proteins and enzymes play in the diseases of interest development, there might also be other positive roles (s) these proteins and enzymes play in the system. With all these, a proper decision can be made on the possibility of achieving a beneficial therapeutic effect without causing a chronic negative outcome to the system.

Protein databases already described above ensure the availability of proteins and enzymes in a format that can be downloaded and utilized for *in-silico* studies. These databases contain proteins and enzymes from humans, animals and different levels of organisms. Some of the databases can be accessed for free, making them open for any interested researcher to access. An interesting feature of most of these proteins available on protein databases like protein data banks is that their active sites are specified, with a ligand molecule attached, making it easier for a specific study to be carried out using the proteins and enzymes.

The natural product databases described above and existing drugs library like drug bank provide the ligands (molecules) which can be utilized or from which new therapeutic inhibitors can be sourced for the purpose of drug repurposing. Because of the number of these natural products as contained in the databases, handling such an enormous amount of data might be challenging, but with the advances in *in-silico* high throughput screening, there are drug design applications that can be deployed in minimizing the number of ligands that could lead to hit molecule(s).

With the advances in computer-aided drug design and bioinformatics, certain steps can be undertaken using natural products towards the discovery and development of better therapeutic inhibitors and also repurposing already existing drugs for the discovery and development of better therapeutic inhibitors. So many studies are already in progress using these steps. There are so many applications that can utilize in the *in-silico* study for the discovery and development of new therapeutic inhibitors. With these applications, drug-likeness and ADMET of thousands of natural compounds can be predicted, protein active site can be established, molecular docking can be simulated, molecular dynamic simulation can be carried out, and *in-silico* that is of the essence in determining which molecule(s) has the lowest chance of failure if taken to in-vitro experiments.

7. Conclusion

Search for discovery and development of new therapeutic inhibitors is an inexhaustible area of research because, even though there are already existing therapeutic inhibitors for different disease conditions, there is always a need for a better option than what is currently available through drug repurposing of already existing drugs or natural products. The abundance of unutilized natural product molecules provides us with a wide range of options from which new and better option therapeutic inhibitors can be sourced. It is well known that the search for the better option is time-consuming and also expensive; there is the need to ensure that the process of discovery and development of new therapeutic inhibitors is undertaken in a manner that minimizes the chances of failure of the process. With the information currently available regarding protein and metabolic pathway databases, ligand and natural product databases, Computer-Aided Drug Design

can be utilized by researchers to initiate steps that will ensure that the most suitable drug repurposing candidates are identified earlier in the process of discovery and development of new therapeutic inhibitor.

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