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Review

Polypharmacology: The science of multi-targeting molecules

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ABSTRACT

Polypharmacology is a concept where a molecule can interact with two or more targets simultaneously. It offers many advantages as compared to the conventional single-targeting molecules. A multi-targeting drug is much more efficacious due to its cumulative efficacy at all of its individual targets making it much more effective in complex and multifactorial diseases like cancer, where multiple proteins and pathways are involved in the onset and development of the disease. For a molecule to be polypharmacologic in nature, it needs to possess promiscuity which is the ability to interact with multiple targets; and at the same time avoid binding to antitargets which would otherwise result in off-target adverse effects. There are certain structural features and physicochemical properties which when present would help researchers to predict if the designed molecule would possess promiscuity or not. Promiscuity can also be identified via advanced state-of-the-art computational methods. In this review, we also elaborate on the methods by which one can intentionally incorporate promiscuity in their molecules and make them polypharmacologic. The polypharmacology paradigm of "one drug-multiple targets" has numerous applications especially in drug repurposing where an already established drug is redeveloped for a new indication. Though designing a polypharmacological drug is much more difficult than designing a single-targeting drug, with the current technologies and information regarding different diseases and chemical functional groups, it is plausible for researchers to intentionally design a polypharmacological drug and unlock its advantages.

1. Introduction

Polypharmacology is the concept that a molecule is designed to bind to two or more targets simultaneously such that the combined effect has a greater therapeutic outcome than binding to just one target. Unlike the conventional drug discovery paradigm of "one drug-one target", polypharmacology is based on the idea of "one drug-multiple targets", where a single drug is designed to act on multiple targets of a single disease pathway or multiple targets involved in multiple diseases [1,2]. Polypharmacology also refers to the ability of a molecule to inhibit targets that produces an effect which is opposite to that of the primary therapeutic target of the molecule, ultimately resulting in a more pronounced therapeutic effect [3].

Conventional research has typically aimed at designing highly specific molecules which exhibit little to no off-target interactions in an effort to minimize the molecule's adverse effects. This approach has found widespread success, especially with diseases which are simple and have a well-established mechanism [4–6]. However, with diseases which are more complex and multi-factorial (e.g., cancer, central nervous system (CNS) disorders and infection), a single target approach is not as effective [4,5,7]. For such diseases, a polypharmacologic drug can be much more useful since it targets multiple proteins and pathways involved in disease onset and development [7]. Advances in medical research have unveiled the etiology of many diseases, as well as identified multiple etiological factors for many diseases, such as schizophrenia, asthma and heart disease [8,9]. These factors all highlight the

Abbreviations: PRMT, protein arginine methyltransferase; BRD, bromodomain; COX-1, cyclooxygenase-1; CysLT1R, cysteinyl leukotriene 1 receptor; DNMT, DNA methyltransferase; EGFR, epidermal growth factor receptor; ENL, erythema nodosum leprosum; GPCR, G-protein coupled receptor; HDACi, histone deacetylase inhibitor; hERG, human ether-a-go-go related gene; KDM, lysine demethylase; KMT, lysine methyltransferase; MKI, multi-kinase inhibitor; PPARγ, peroxisome proliferator-activated receptor-γ; PROTAC, proteolysis-targeting chimera; ROCS, rapid overlay of chemical structures; ReDO, repurposing drugs in oncology; ReFRAMEdb, repurposing, focused rescue, and accelerated medchem database; SEA, similarity ensemble approach; sEH, soluble epoxide hydrolase; STS, steroid sulfatase; SARM, steroidal androgen receptor ligand; TNF-α, tumor necrosis factor α; UPS, ubiquitin-proteasome system; VEGFR2, vascular endothelial growth factor receptor 2.

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great need for polypharmacologic drugs, and recently there has been a steep rise in their development [10].

2. The need for polypharmacology

Single target drug discovery has long been the primary aim of pharmaceutical research. This approach has been successful in discovering many single target drugs while preventing adverse effects due to "off-target" interactions. Despite these advances, there has been an increase in the rate of drug attrition over the past few decades, primarily due to molecules being sub-efficacious. Additionally, retrospective analyses of a few successful drugs revealed them to be acting on multiple targets, indicating their therapeutic efficacy is likely linked to their multi-targeting nature [11]. This strongly suggests that sub-efficacy attritions are related to the single targeting nature of drug discovery. It is for this reason that many research groups are now opting to design multi-targeting drugs, considering the numerous advantages it has over conventional single-target drug design.

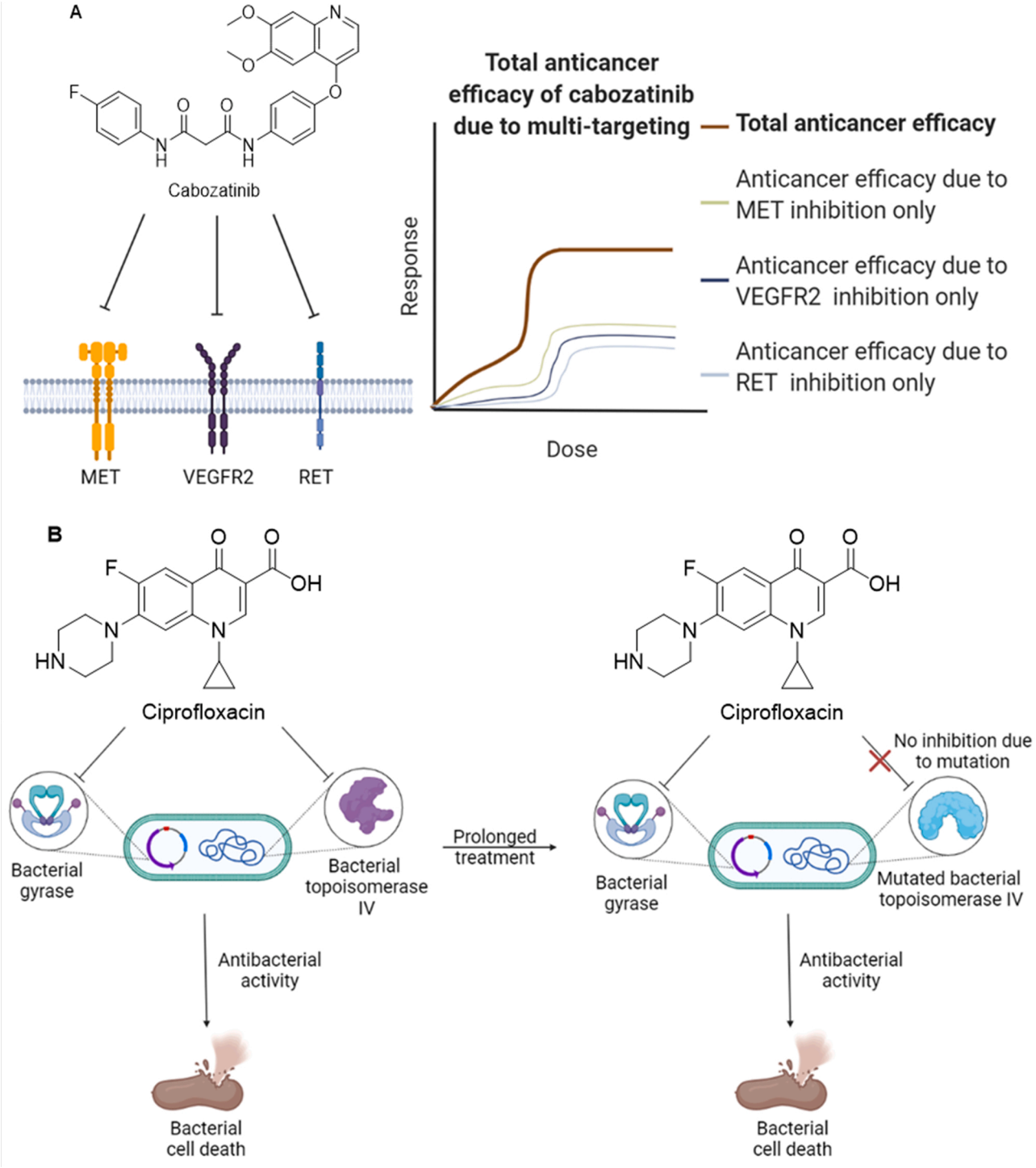

Fig. 1. Advantages of polypharmacology. A) Arbitrary representation of higher total anticancer efficacy of the MKI cabozatinib compared to the efficacy due to inhibition of each of its individual targets. B) Polypharmacological action of the fluoroquinolone ciprofloxacin delays development of antimicrobial resistance. Created with Biorender.

In general, the limits of single-target molecules have been demonstrated. In yeast, inhibiting 85–90% of the known individual targets resulted in no observable effect. Additionally, mouse knockout studies demonstrated that only 10% of all targetable genes were effective as single therapeutic targets [12,13]. These results show that despite a drug being highly specific for its intended target, it may fail to achieve the necessary therapeutic effect, thereby demonstrating the need for a polypharmacological approach.

As previously stated, multi-targeting molecules are imperative to drug development in complex and multi-factorial diseases. Tackling such diseases requires simultaneous action on multiple targets to illicit a greater therapeutic effect. This can be achieved either by incorporating multiple drugs in the treatment regimen, each targeting a specific single pathway (polypharmacy), or by using a single polypharmacologic drug. Though polypharmacy helps in managing multiple chronic diseases through enhanced therapeutic efficacy and improving a patient's quality of life, there are numerous advantages of a polypharmacologic approach over a polypharmacy approach [14]. Specifically, polypharmacology reduces the treatment complexity, pharmacokinetic complexity, and drug-drug interactions, while also improving patient compliance. Simultaneous action on multiple targets increases the therapeutic efficacy through an additive effect, or synergism. One particular example of synergism through polypharmacology is found in cancer treatment where multi-kinase inhibitors (MKIs) have been widely used due to their action on multiple processes governing proliferation, angiogenesis, and migration [12,15]. Kinase inhibitors have a high potential to multi-target due to a highly conserved adenosine triphosphate (ATP) binding site across all kinases. Lapatinib was an early marketed MKI optimized to target epidermal growth factor receptor (EGFR) and ErbB2, while concurrently excluding the undesirable effects of previously designed MKIs (e.g., imatinib) [16]. Similarly, Fallahi et al. reported the use of the MKI cabozatinib, which targets MET, vascular endothelial growth factor receptor 2 (VEGFR2) and rearranged during transfection (RET), in multiple cancer types (Fig. 1A) [17]. This synergistic multi-targeting of proteins constitutes network pharmacology. In network pharmacology, a disease's signaling pathways are mapped out, and certain nodes (proteins) on the mapped network, which could belong to the same or different signaling pathway, are targeted by a single molecule [13,18]. This concept of network pharmacology is further discussed later in this review.

In both anticancer and antimicrobial therapies, one of the major reasons conventional therapies have failed is due to drug resistance. Tumor and microbial cells evolve with time and develop resistance through many factors, such as mutation and upregulation of the target, as well as drug efflux [12,19,20]. These resistance mechanisms usually arise when only a single protein, or pathway, has been modulated, making a highly effective drug less efficacious. With polypharmacology, targeting many disease pathways at once using a single molecule has a great potential for overcoming, or at least delaying, drug resistance [21,22]. The targeted cells are unable to restore all affected pathways at once, and the polypharmacologic drug continues to be efficacious, thereby minimizing drug resistance. For instance, He et al. demonstrated that the molecule AEE788 simultaneously targeted EGFR/HER2 and VEGFR, which resulted in triple negative breast cancer cells overcoming resistance typically observed for mTOR-targeted therapy [23]. Drug resistance is widely prevalent in infections as well. For this reason, antibiotics are designed to have multiple targets, such as fluoroquinolones, which act on bacterial gyrase and topoisomerase IV, aiding in its ability to overcome resistance (Fig. 1B) [12,20].

Since selective drugs act on a single target, they are perceived to be intrinsically safer, with fewer adverse effects. However, many adverse effects originate from an interaction with the therapeutic target itself. These target-related adverse effects account for about a quarter of all drug withdrawals [12]. In contrast, when multiple targets are modulated through a single polypharmacologic molecule, only the therapeutic effect is synergized and not the adverse effects, as is the case for polypharmacy. This "selective synergism" is evident when multiple targets are exclusive to diseased cells and not healthy cells [12,24,25]. The potent analgesic, tapentadol, is such an example, as it acts through both μ opioid receptor agonism and norepinephrine reuptake inhibition. This dual action makes it safer and possess fewer adverse effects as compared to conventional opioids (e.g., morphine) due to its specific targeting of the μ opioid receptor. Despite the equianalgesic dose of tapentadol being higher than that of morphine, tapentadol's selective synergism (due to its dual-targeting nature) results in much more benign side effects than morphine [12,26,27].

CNS disorders such as schizophrenia and Alzheimer's are both complex and multi-factorial, and their treatment is typically associated with an array of adverse effects [28,29]. One way of increasing the net efficacy of their therapy is by modulating the target protein while simultaneously inhibiting pathways that are responsible for the adverse effects. This usually cannot be achieved with drugs that are highly specific for a single target and often results in discontinuation of the therapy due to its severe adverse effects. For example, the anti-obesity serotonin reuptake inhibitor chlorphentermine was withdrawn from the market due to its cardiotoxicity [30]. In the case of first-generation typical antipsychotic drugs like chlorpromazine, inhibition of the dopamine D2-like receptor in the nigrostriatal pathway is often associated with extrapyramidal motor side effects, such as dystonia and akathisia (Fig. 2) [7,29,31]. One way of reducing the side effects is by introducing anti-serotonergic 5-HT_{2A} activity. This is achieved by the second-generation, atypical antipsychotic drug aripiprazole, which has agonistic activity on the dopamine D2, D3 and serotonin 5-HT_{1A} receptors, as well as antagonistic activity on the serotonin 5-HT_{2A} receptors. However, the D2 agonistic activity of aripiprazole was associated with adverse events (AEs) such as akathisia, insomnia, restlessness, agitation, and nausea (Fig. 2) [32,33]. This led to the development of third-generation, atypical brexpiprazole, which has partial agonistic activity at the D2 and serotonin 5-HT_{1A} receptors, antagonistic activity at 5-HT_{2A}, and noradrenaline $\alpha_{1B/2\,C}$ receptors, while also exhibiting similar affinity for each of these receptors [33–35]. Compared to aripiprazole, brexpiprazole has lower intrinsic activity at the D2 receptor and therefore fewer D2 agonism related AEs. It also exhibited greater antagonistic activity at 5-HT_{2A} than aripiprazole, which might be responsible for the reduced D2 inhibition-mediated akathisia [33,36]. Furthermore, brexpiprazole demonstrated a greater affinity for the serotonin 5-HT_{1A} and adrenergic α_1 receptors resulting in improved depressive symptoms (5-HT_{1A}) and extrapyramidal motor side effects (α_1) [33,37–40]. This well balanced activity allows brexpiprazole to decrease symptoms of schizophrenia while simultaneously mitigating the risk of weight gain and extrapyramidal motor side effects [7,41]. The improved efficacy of brexipiprazole results in lower daily dose which would further attenuate its side effects (Fig. 2) [42].

Lastly, designing a multi-targeting drug is more economical, considering the numerous clinical trials that need to be performed for each individual drug and its respective target [7]. These advantages provide a strong argument in favor of polypharmacology over conventional single target therapy. In order to incorporate polypharmacology into drug design, one needs to fully understand the inherent complexity of multi-target design and follow a systematic approach to achieve the desired outcomes.

3. Polypharmacology based drug designing

3.1. Antitargets – the mines to avoid

In order to be polypharmacologic, a molecule needs to possess all the structural features required for it to act on multiple targets. The ability of a molecule to bind to multiple proteins within a disease pathway is called "promiscuity". It is the intrinsic property which makes a molecule polypharmacologic. Despite promiscuity being closely associated with polypharmacology, the term often has a negative connotation to it,

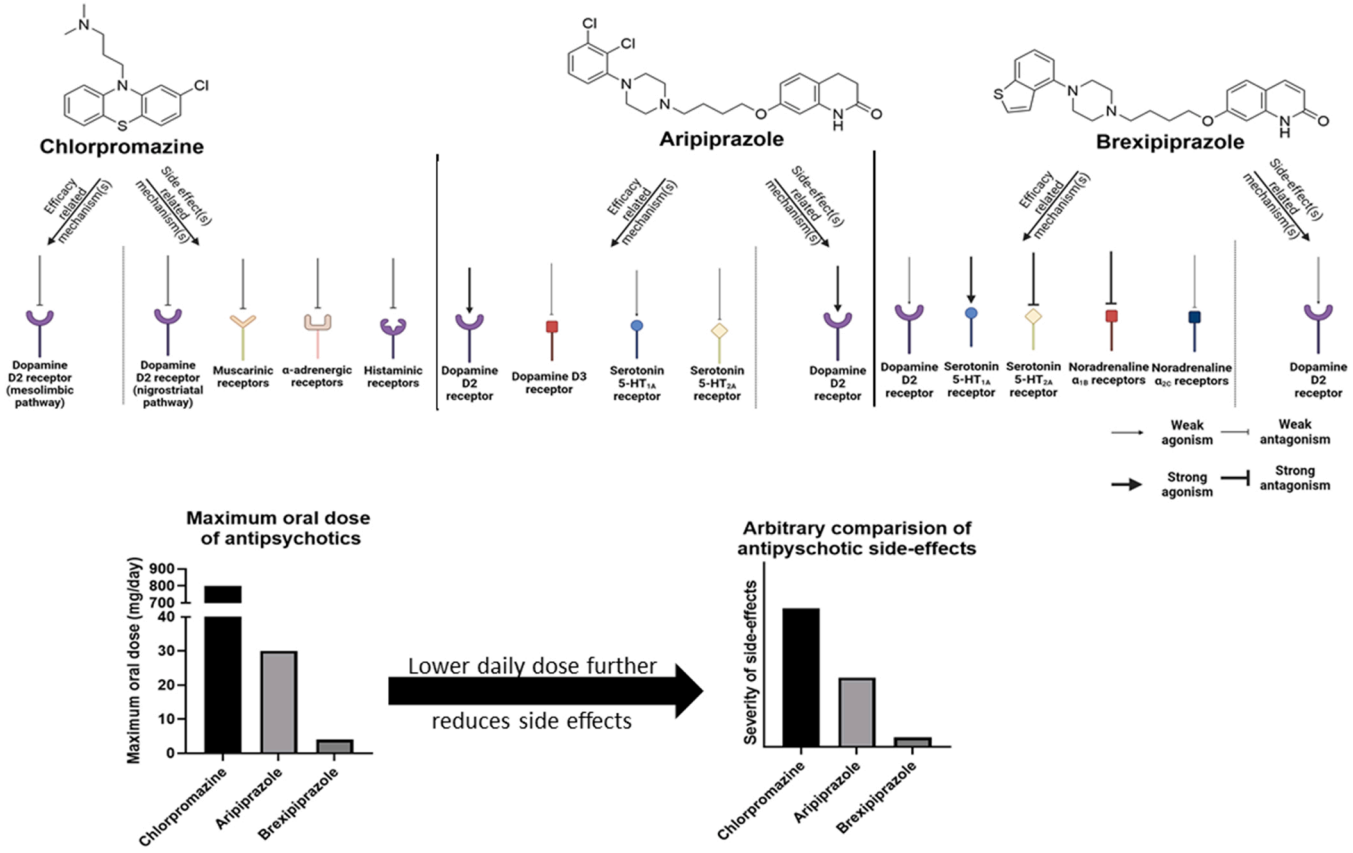

Fig. 2. Selective synergism of brexipiprazole resulting in lower daily dose which further reduces side effects. Created with Biorender and GraphPad Prism.

implying it binds to off-targets resulting in adverse effects. Therefore, for a molecule to become polypharmacologic, it needs to be selectively promiscuous, such that it binds to multiple therapeutic targets, while avoiding off-target effects. In order for a polypharmacologic molecule to be effective and devoid of off-target effects, the molecule should demonstrate minimal binding to an "antitarget" (Fig. 3). One method for identifying potential binders of an antitarget is by a preliminary screen of a library of molecules for antitarget binding. This would not only protect volunteers participating in clinical trials, but also reduce the likelihood of future attrition or drug withdrawals. Additionally, early screening would reduce the need to modify a well-optimized molecule at a later stage, which is a major setback for any drug discovery research group [12,43]. For this reason, research organizations have identified up to 180 safety-related antitargets, against which they evaluate their new molecules [12]. Testing service against a list of antitargets is provided by Eurofins discovery (https://www.eurofinsdiscoveryservices.com/services/safety-and-efficacy/safety-pharmacology/safety-panels).

To avoid redundancy, research organizations have short-listed a smaller group of antitargets for screening their molecules against at an early stage. This will help in identifying promiscuous molecules as well as predict antitarget binding related adverse effects. Scientists from AstraZeneca, GlaxoSmithKline, Pfizer and Novartis collaboratively identified 44 targets and proposed them as a "minimal panel" for early safety screening (published by Bowes et al.) [12,44]. This group contains only key and well established antitargets which have a high hit rate and severe adverse effects [12,45]. Some of the most encountered antitargets, their hit rates, and their associated adverse effects are listed in Table 1.

To predict antitarget-related adverse effects, one cannot reliably use the preclinical data due to species-related differences. For example, rodent models do not accurately predict cardiovascular side effects observed in humans. This is primarily due to different ion channel contributions in humans as compared to rodents [12,47]. For this reason, the international council for harmonization (ICH) S7A guidelines for safety screening recommends that ligand binding or enzyme assays are used to determine antitarget binding related adverse effects [12,48]. Additionally, radioligand displacement assays and functional assays are widely used for safety panel screening (Fig. 3). However, for certain G-protein coupled receptors (GPCRs), functional assays are preferred for two reasons. First, radioligand displacement assays cannot differentiate whether an interacting molecule is an agonist or an antagonist. Second, functional assays are better predictors for adverse effects, since for many receptors, adverse effects are only related to agonistic activity rather than antagonistic activity [12,45]. These in vivo and in vitro methods, however, are time-consuming and costly, and for this reason there is an increase in the use of *in silico* computer-aided drug design methods to predict interactions (Fig. 3). Though not as accurate, *in silico* methods are less expensive, less time consuming, and help to eliminate molecules with poor pharmacokinetic properties, reducing the risk of failure in the clinical trials [49,50]. They are mainly data driven and are based on the available knowledge of the molecules, the targets, and their interactions [51]. The *in silico* computational methods used to identify promiscuity and predict antitarget interactions are described in detail in Section 3.2.3 of this review.

Apart from antitarget interactions, evaluating the pharmacological profile of a molecule also aids in predicting the potential adverse effects of the molecule. If a new molecule has the same pharmacological targets as that of a known drug, it is also highly likely to have similar adverse effects [12,52–54]. Together, antitarget and pharmacological profile screening help in predicting promiscuity and the potential adverse effects of a molecule. Additionally, there are certain molecular features such as lipophilicity and ionization state, which if present, could predict promiscuity, thereby predicting polypharmacology.

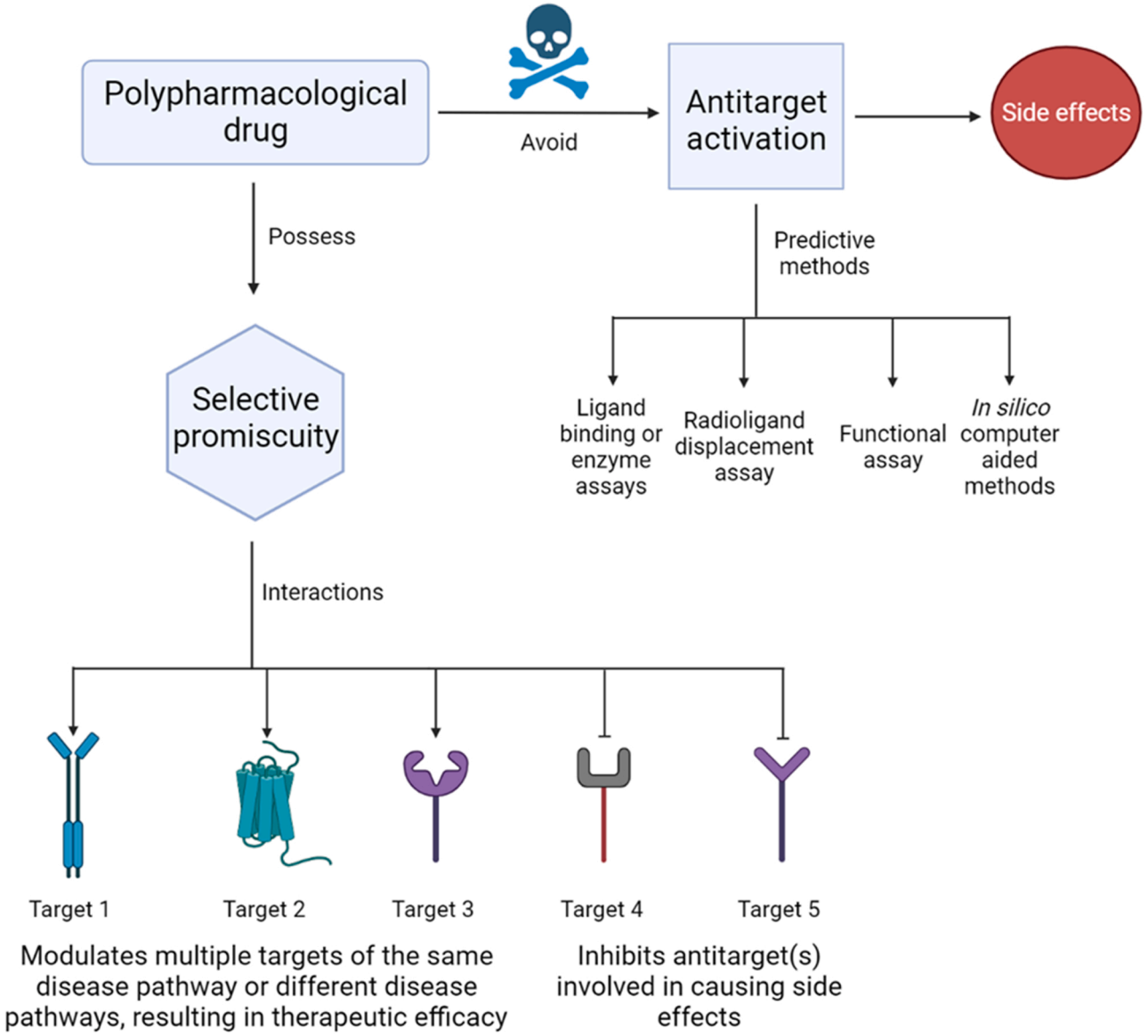

Fig. 3. Selective promiscuity requirement for polypharmacology. Created with Biorender.

3.2. *Identifying promiscuity*

As previously discussed, in the era of single targeting drug development, promiscuous drugs were also often discovered. Although these drugs were designed with the intention of being single targeting, many were recently identified to be serendipitously polypharmacologic. As polypharmacology has become more popular in drug development, a molecule's promiscuity is often discovered in the later stages of biological screening. Once promiscuity is discovered, the molecule is then rationally optimized for increased therapeutic efficacy through multi-targeting. This process of optimizing the molecule at a later stage is often more time consuming and costly. Medicinal chemists have now identified which molecular features tend to make a molecule promiscuous (discussed in detail later) [55]. These molecular features coupled with various drug design techniques aid in early promiscuity identification and in developing a multi-target lead.

3.2.1. *Physiochemical properties to identify promiscuity*

Since 2006, numerous studies have linked promiscuity to specific physicochemical properties, such as lipophilicity, ionization state, and molecular weight (Fig. 4). In general, lipophilicity is considered a strong predictor of pharmacological promiscuity since most protein binding sites are hydrophobic, and lipophilic interactions are major contributors to ligand affinity. Therefore, highly lipophilic molecules tend to be more promiscuous. A library of molecules from various pharmaceutical companies has demonstrated that pharmacological promiscuity increased significantly with a cLogP greater than 2 [12,55].

Interestingly, molecules with similar lipophilicities often vary greatly in their degree of promiscuity. Additionally, pKa appears to play a secondary role in influencing promiscuity when lipophilicity was similar. For example, basic molecules exhibited a sigmoidal relationship between promiscuity and ClogP, with an inflection point at ClogP $\sim$ 2. However, for neutral molecules, the sigmoidal relationship was less obvious with only a moderate increase in promiscuity in relation to ClogP. Additionally, high promiscuity was frequently observed for neutral molecules at ClogP $>$ 1 [55]. This demonstrates that although lipophilicity is an important predictor of promiscuity, it is not the only predictor, and other molecular properties need to be considered. [12, 55–58].

In addition to lipophilicity, the ionic state of a molecule also has a substantial influence on promiscuity (Fig. 4). Compounds which are ionized at physiological pH may not naturally exhibit promiscuity. This

Table 1
[a] Frequently encountered antitargets, their hit rates, and associated adverse effects [12,46].

Antitarget	Hit rate (%)	Associated adverse effects
Human ether-a-go-go related gene (hERG) channel	–	Arrythmia
Serotonin 5-HT_{2B}	14	Agonist binding: valvulopathy, pulmonary hypertension
Serotonin 5-HT_{2A}	11	Agonist binding: cognitive impairment, hallucination
α_{1A} adrenergic receptor	10	Agonist binding: arrythmia Antagonist binding: orthostatic hypotension
Dopamine D_2 receptor	9	Agonist binding: confusion, emesis Antagonist binding: orthostatic hypotension
Histamine H_1 receptor	6	Antagonist binding: weight gain, sedation, somnolence
α_{2A} adrenergic receptor	6	Agonist binding: hypotension, sedation
Dopamine D_1 receptor	5	Antagonist binding: dyskinesia, tremor
Muscarinic M_{1-5} receptors	5	Adverse cardiovascular and metabolic effects, cognitive impairment
μ- opioid receptors	3	Agonist binding: sedation, respiratory depression, abuse liability

[a] Hit rate in the table is the percentage of druglike compounds which bound to the respective target with an $IC_{50} < 1$ μM in the BioPrint data set.

appears to be due to the specific distance and angular requirements necessary for hydrogen bonding and other polar interactions to take place [55,59]. An ionized molecule which is not properly oriented or is not close enough to the oppositely charged amino acid residue on the target, would fail to interact with the target through hydrogen bonding and other polar interactions. This would render the molecule to be less promiscuous. However, basic molecules (pKa > 6) which are usually protonated at physiological pH, frequently exhibit promiscuity in safety screens, especially if they have two or more aromatic rings close to the basic center (e.g., acridines) [56]. Moreover, data from the BioPrint database revealed that bases, including quaternary bases with a positive charge, exhibited more promiscuity than acids, neutral compounds, zwitterions, and uncharged bases [55,58].

The promiscuity observed for basic molecules is primarily due to the positive charge at the basic center being a common aspect of multiple pharmacophores with various targets (e.g., aminergic GPCRs non-aminergic GPCRs, and ion channels) [12,56]. However, a high percentage of these targets (~15–25%) are antitargets, and the positive charge may be perceived as a promiscuity liability. Aminergic antitargets also have a high hit rate for compounds with a basic center, such as the serotonergic 5-HT_{2B}. In the BioPrint database, it is observed that aminergic antitargets bind to more than one third of basic compounds with sub-micromolar affinity [12,55,60]. In a safety screening of Roche compounds, a positive charge was also found to be the most important determinant of off-target interactions [55,61].

The effect of molecular weight on promiscuity has also been studied (Fig. 4). In general, molecular weight reflects a molecule's complexity, as higher molecular weight compounds are typically more complex. Therefore, the simplicity of a low molecular weight compound may increase the chances of surface complementarity with the binding site, making it more promiscuous. On the other hand, a higher molecular weight compound could have a higher chance of possessing the pharmacophoric features of the protein binding site. One particular example of this is peptidic hormones, where only a small portion of the molecule is responsible for its pharmacological interaction [55,62]. These contradictory aspects have also been observed experimentally. In Pfizer's high throughput screening (HTS) data, an inverse relationship between molecular weight and promiscuity was observed, whereas, in the case of Novartis' safety screening data, higher molecular weight molecules were found to be more promiscuous [63,64]. Therefore, molecular weight does not seem to be a useful predictor of promiscuity.

Lipophilicity and ionization state are good predictors of promiscuity, while other molecular features are less effective. These less effective molecular features include: number of rotatable bonds, ring count, and number of ring assemblies (all of which often have a positive correlation with promiscuity) (Fig. 4). The effect of these molecular features on

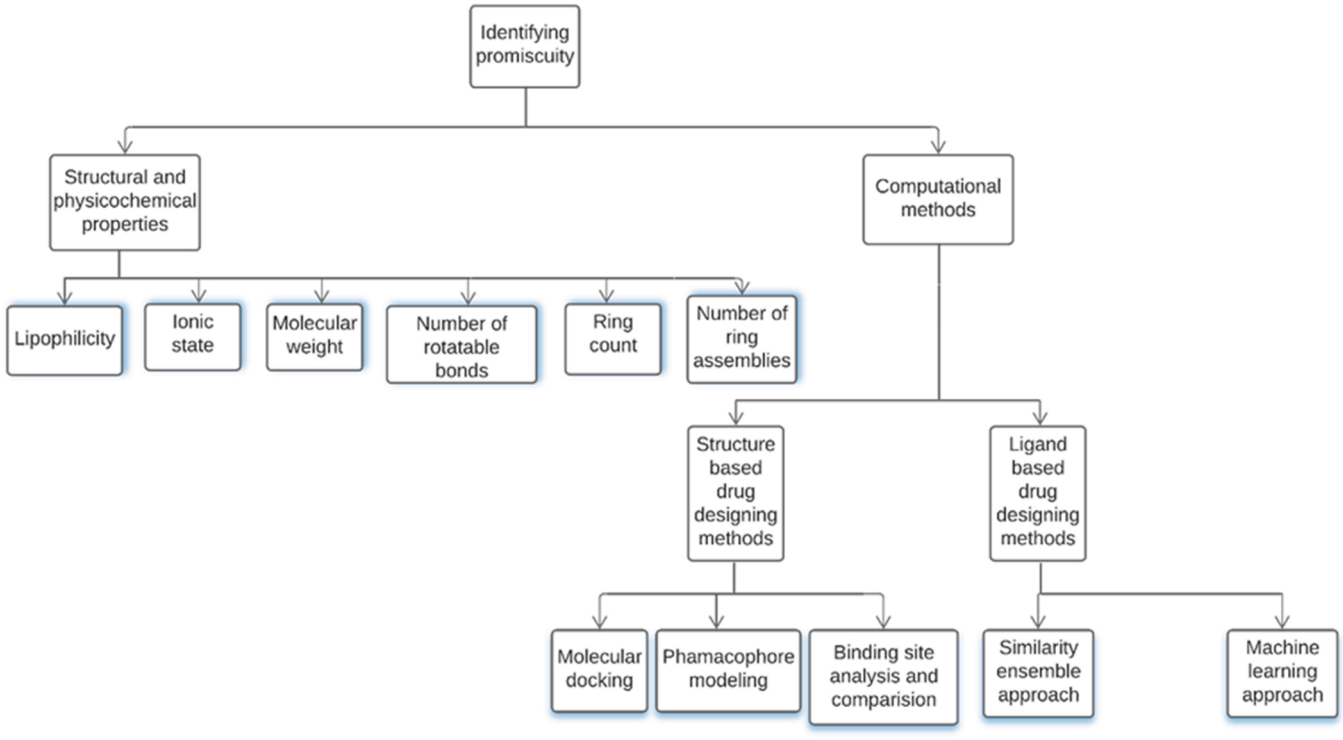

Fig. 4. Criteria and methods to identify promiscuity. Created with Lucid Charts.

promiscuity is indirect and correlates with their relationship to lipophilicity, which could be the principal underlying determinant [55,63]. Similarly, molecules with low polar surface area and fewer hydrogen bond donors/acceptors have higher lipophilicity, and are also more promiscuous [56,58,65,66]. Furthermore, molecules which have little flexibility and numerous side chains are often less promiscuous, as these molecules require high shape complementarity for them to bind to the desired protein binding site [12,56,58,66–68].

3.2.2. Structural features to identify promiscuity

One reason why a molecule is promiscuous is its pharmacophoric similarity within a target protein family as well as between unrelated proteins [12,69]. Two such protein families which are associated with promiscuity and have pharmacophoric similarities are aminergic GPCRs and kinases. It has been established that basic amines (secondary or tertiary) connected to an aromatic ring via a 2–5 atom linker forms the prototypical pharmacophore of aminergic GPCRs and ion channels. Therefore, molecules possessing such features are highly likely to be promiscuous [55,70,71]. Similarly, the "2–0" rule helps to identify molecules which are likely to have kinase activity. The rule involves counting certain structural fragments: more than 2 heteroaromatic nitrogens (e.g., N or NH), or more than 0 aromatic NH substituents and nitriles. Comparatively, the presence of a 'heteroaryl-NH-aryl' motif is much more predictive of kinase activity [12,72].

The presence of these molecular and structural features is only predictive of promiscuity. In order to determine which targets the molecule interacts with, one needs to study existing chemical and biological databases. However, these databases are extremely large, as they incorporate all information related to chemical scaffolds and biological proteins. Therefore, relating the chemical and biological spaces is highly complex. To resolve this complexity and best predict the relationship between two spaces, efficient computer-based techniques have emerged. Along with current advancements in technology, there has been an increase in the utilization of *in silico* computational methods in drug discovery to predict an interaction between a molecule and a protein [46].

3.2.3. Computational methods to identify promiscuity

State-of-the-art computational methods have made it possible to predict the interaction of a molecule with a panel of targets, allowing us to anticipate promiscuity, antitarget interaction, and a molecule's pharmacokinetic properties. This can be achieved during the pre-synthetic iterative design and optimization phase. With numerous techniques involved, computational methods can broadly be classified into: statistical data analysis and bioinformatics, structure-based approaches, and ligand-based approaches; with each having its own set of advantages and limitations [6,51,73]. Table 2 summarizes the computational methods used to identify promiscuity.

3.2.3.1. Structure based drug designing (SBDD). SBDD methods, also known as target-centric methods, study the 3-D structure of the macromolecular target (typically a protein or ribonucleic acid (RNA)) and are based on the premise that a set of proteins with similar structure would have similar selectivity properties and would therefore bind to similar compounds. It identifies key sites and interactions which would

Table 2
Summary of computational methods used to identify promiscuity.

Computational Method	Description	Advantages	Limitations
Structure based drug designing (SBDD)	Studies the 3-D structure of the target protein. Based on premise that proteins with similar 3-D structure will bind to similar molecules	- Time and cost effective - Molecular docking accurately predicts promiscuity by analyzing the steric and electrostatic complementarity of the ligand and its binding energy with the binding site - Overcomes false positives and negatives associated with a chemical similarity approach - Binding site analysis and comparison helps in filling in residues in a protein of interest by grafting of the missing residues from closely related structures	- Only used for proteins with known crystal structures - Results are affected by differences in the conformations of the binding site residues
• Molecular docking	Predicts the degree of interaction between the protein target and the molecule based on spatial shape complementarity and energy matching. Virtually evaluates the binding interaction of a molecule with the 3-D crystal structures of various protein targets		
• Pharmacophore modeling	Determines the potential of a single molecule to possess the pharmacophoric features needed to interact with different proteins. Online database: PharmMapper (http://www.lilab-ecust.cn/pharmmapper/)		
• Binding site analysis and comparison	Systematically compares the binding sites of a group of proteins and identifies groups of proteins with similar binding sites which potentially share a common ligand. Online database: BioGPS (http://biogps.org/)		
Ligand based drug designing	Uses the structure of a known ligand to identify the key structural features necessary for target binding. Based on the assumption that molecules with structural or chemical similarity to the known ligand would likely bind to biologically relevant protein targets	- Do not require the target protein crystal structure - Can evaluate natural products or substrate analogues that interact with the target and produce the desired pharmacological effect - Machine learning algorithms do not assume any relationship between the properties of the target and the molecular descriptors	- Cannot be used for protein targets which have no known ligand - Could give false positives and negatives based on their similarities and differences with the know ligand - In machine learning algorithm a single misclassification in the training dataset could result in incorrect prediction of a new molecule
• Similarity ensemble approach	3-D structure of the molecule is compared to that of the known ligand of different targets to determine if it possesses the necessary pharmacophoric features. Uses the rapid overlay of chemical structures (ROCS) program		
• Machine learning approach	Uses regression algorithms to classify compounds into categories such as active or inactive and rank them based on their probable activity		
• Quantitative flux modeling	Computes the degree of molecular modulation required to produce the desired therapeutic effect with minimal side effects. Predicts that maximum therapeutic efficacy would be achieved by partial modulation of target combinations rather than complete modulation a single target		

result in their pharmacological function. In order to use SBDD, the 3-D structure of the target needs to be identified. [51,74]. Once identified, molecular modeling software can be used to investigate the physicochemical properties of the drug binding site. Properties such as hydrophobicity, polarity, hydrogen bonding capacity, electrostatic field, as well as the key binding amino acid residues are analyzed. The chemical database is then searched to identify molecules which have shape complementarity to the binding site and have high binding affinity. This can be useful in virtually identifying multiple biological targets for the same chemical structure, thereby predicting promiscuity and polypharmacology. The selected molecules are then synthesized and their pharmacological activity is evaluated [75].

3.2.3.1.1. Molecular docking. SBDD can be achieved through numerous methods such as molecular docking, pharmacophore modeling, and binding site analysis and comparison (Fig. 4) [73]. Molecular docking helps in predicting the degree of interaction between the protein target and the molecule based on spatial shape complementarity and energy matching. The inherent promiscuity within a chemical structure can be identified by virtually evaluating its binding interaction with the 3-D crystal structures of various protein targets [75].

3.2.3.1.2. Pharmacophore modeling. Another method of achieving SBDD is by pharmacophore modeling. Pharmacophore is the term which describes the molecular features necessary for a ligand to interact with a biological macromolecule and illicit a response. This essential requirement of molecular features (hydrogen bond donors, hydrogen bond acceptors, aromatic moieties, etc.) can be fulfilled by a variety of functional groups, and many different molecules which possess these essential features would successfully interact with the protein. In pharmacophore modeling, the potential of a single molecule to possess the pharmacophoric features needed to activate different proteins is determined. An online database called PharmMapper uses a reverse pharmacophore matching approach and identifies the potential protein targets for a test molecule. This leads to the identification of all proteins whose pharmacophoric requirements are fulfilled by the molecule and to which the molecule is expected to bind promiscuously [76–78].

3.2.3.1.3. Binding site analysis and comparison. Promiscuity in a molecule can also be identified using SBDD by a binding site analysis and comparison approach. As the name suggests, this method involves a systematic approach comparing the binding sites of a group of proteins. This comparison eventually helps in identifying a group of proteins that could share a ligand, suggesting the promiscuous nature of those ligands [79]. To achieve this, a fast and semi-automated approach called BioGPS can be used which characterizes binding cavities using molecular interaction fields [80]. Duran-Frigola et al. identified 87,300 binding cavities in 31,900 protein chains from 3700 unique proteins using BioGPS. They then compared the binding pockets and classified pockets as 'similar' if they had a score of 0.6 or above. The similarity of the binding pockets was then confirmed by analyzing the co-crystallized ligands. It was observed that pairs of binding pockets with a score above the cutoff accommodated the same ligands, confirming the applicability of the approach in identifying promiscuity [79].

A recent advancement to the binding site analysis and comparison method is protein-ligand interaction fingerprints (IFS). IFS rescores docking results and is more suited to cases where the proteins have remote similarities, which is when the whole proteins are aligned or only their binding sites are aligned. In IFS, the identification of individual amino acids involved in binding is ignored. Instead, it focuses on the interaction that exists between the receptor and the ligand, thereby providing a more holistic similarity evaluation of distantly related proteins [51]. It has been shown that IFS is more effective in computing similarity than binding site analysis and comparison, and it is more useful in handling large datasets [51,81].

In general, SBDD is a powerful computational technique which is both time- and cost-effective in developing new drug lead molecules as well as identifying promiscuity [82]. By utilizing the 3-D structure of the target and analyzing the steric and electrostatic complementarity of the ligand with the binding site, SBDD helps in accurately predicting promiscuity. It overcomes the false positives and negatives associated with a chemical similarity approach, where a molecule is predicted to bind to a target if it has structural similarity to its known ligand. In addition, SBDD helps in assessing binding site structural similarities of various targets and in identifying potential protein-ligand interactions at critical nodes of a disease pathway. However, SBDD has its limitations. It can only be used for proteins with known crystal structures and results are affected by differences in the conformations of the binding site residues, which is difficult to predict [73,83].

3.2.3.2. Ligand based drug designing. In contrast to the previously described methods, ligand-based computational methods do not have the prerequisite of a target protein crystal structure. These methods, also known as compound-centric approaches, are based on the assumption that molecules with structural or chemical similarity to the known ligand would likely bind to biologically relevant protein targets [51,73,78]. Using the structure of the known ligand, a pharmacophoric model is derived which identifies the key structural features necessary for target binding. A molecule possessing these critical structural features is assumed to be binding to the biological target of the known ligand [75]. It is similar to the pharmacophore modeling method used in SBDD, however, the chemical database is searched to identify which known pharmacophoric features the new molecule possesses. This approach is also useful in predicting promiscuity, as a molecule might simultaneously possess the critical pharmacophoric features of two or more targets, rendering it polypharmacologic [73,78].

3.2.3.2.1. Similarity ensemble approach. Ligand-based approaches often use a method known as the similarity ensemble approach (SEA), where the 3-D structure of the molecule is compared to that of the known ligand of different targets to determine if it possesses the necessary pharmacophoric features (Fig. 4). The method uses the 3-D-shape similarity analysis program, rapid overlay of chemical structures (ROCS), to predict which protein targets a molecule would bind based on its shape similarity to the known ligand. This generates a pharmacological profile for the molecule [12,78,84,85]. The SEA has become increasingly popular, and it has successfully predicted the potential targets of multiple molecules. For example, it was successfully predicted that the synthetic estrogen molecule chlorotriazine would inhibit cyclooxygenase-1 (COX-1). This provided an explanation for the abdominal pain side-effect observed from chlorotriazine treatment [12,86–88].

3.2.3.2.2. Machine learning approach. Machine learning (ML) approaches have recently been developed as a ligand-based approach for target prediction (Fig. 4). It makes use of regression algorithms such as support vector machines (SVM), decision trees (DT), k-nearest neighbor, naïve Bayesian models, and artificial neural networks to classify compounds into categories (active or inactive) and rank compounds based on their probable activity [51,89,90]. The reliability of the predictions made by these algorithms using ML approaches can be further improved if combined with specific molecular features from both the target and the compound which are involved in the drug-molecule interaction [51]. In polypharmacology, multi-target activity evaluation requires methods for efficient target deconvolution. This is especially important in protein families which have similar structures and sequence domains and are also highly targeted (e.g., kinases). Using ML approaches, it is possible to utilize the information gained from similar kinases and compounds to predict the activity of currently unexplored compound-kinase interactions [91–93]. Quantitative results are then improved when ML approaches are used along with the Illuminating the Druggable Genome (IDG) consortium (https://druggablegenome.net/) - a National Institutes of Health (NIH) Common Fund program with an objective to improve the understanding of understudied proteins within three drug-targeted protein families: G-protein coupled receptors, ion channels, and protein kinases. The program specifically aims at improving

the targeting of understudied kinases by kinome-wide profiling small-molecule agents, with the aim of further exploring the activity profile for the understudied human kinome [91,94,95].

In studies where specific disease models are available, the results obtained by using ML algorithms can be enhanced by analyzing the drug response profiles with molecular and genomic profiling of the disease models (e.g., copy number variation, proteomic, transcriptomic, methylation, and exome and RNA-Seq datasets) [51,96]. Compared to other computational methods, ML approaches are novel and have been increasingly applied with greater accuracy to serve a wide range of objectives, such as classifying molecules as potent inhibitors and non-inhibitors of cytochrome P450, and predicting the antitarget interactions of tivozanib, an investigational VEGFR inhibitor [51,97,98]. Recent works by Lavecchia and Cichońska et al. provide a comprehensive guide describing ML algorithms and their applications [90,91].

As previously discussed, the main advantage of a ligand-based approach is that it does not require the crystal structure of the protein. However, it needs a reference ligand structure to compare with. Therefore, it cannot be used for protein targets which have no known ligand. Additionally, new molecules which are too dissimilar in structure compared to the existing ligands might be anticipated to be "non-interacting" with a protein target. This could result in a false negative, as all active chemical structures for a protein target may have not been identified. Predicting the polypharmacological profiles of such molecules would be very difficult. On the other hand, new molecules which are very similar in structure to the known ligand could provide a false positive if they differ at the key positions involved in the target interaction. In such cases, SBDD would be more useful and accurate by determining the steric and electrostatic complementarity of the molecule to the target binding site. In general, since both SBDD and ligand-based methods have their own set of advantages and limitations, using them in conjunction would be more accurate and provide more robust results towards identifying polypharmacologic molecules.

In addition to aiding in the identification of suitable target combinations as well as predicting ligand promiscuity, *in silico* quantitative flux modeling computes the degree of modulation required to produce the desired therapeutic effect with minimal side effects. This theory of targeting multiple pathways with low drug doses predicts that maximum therapeutic efficacy would be achieved by partial modulation of target combinations rather than fully modulating a single target [7,99]. Using time resolved flux analysis, Yang et al. studied cyclooxygenase and lipoxygenase (the individual branches of the arachidonic acid metabolic network) in human polymorphous leukocytes and predicted the optimal target combinations needed for synergistic effects. These were then validated by time-resolved LC-MS/MS profiling of pro- and anti-inflammatory metabolites of arachidonic acid [7,100,101].

Despite computational methods being quick and economical, they only provide a hypothesis which needs further experimental validation to confirm promiscuity. Since computational approaches also rely on very heterogeneous data, the use of focused in vitro experiments can help in achieving more reliable results. On the other hand, detailed *in silico* analysis of the targeting pathways helps in reducing the influence of potential off-target activities of the screened ligands. Therefore, the combined use of computational and experimental approaches would provide more accurate data in identifying synergistic target combinations and in confirming promiscuity [7]. The recent increase in the use of computational strategies to predict polypharmacology suggests that combining both experimental and computational methods would likely be the future of polypharmacological drug design [73].

The molecular features and computational methods described above would help in predicting whether a designed molecule would exhibit polypharmacology. However, to intentionally incorporate promiscuity in a molecule, one needs to first identify the molecular target combinations and then follow a systematic designing approach which would integrate the pharmacophoric features of the chosen targets.

3.3. Incorporating polypharmacology

3.3.1. Target selection

The numerous advantages of polypharmacology over single-targeting drugs and polypharmacy makes it a very promising avenue for drug design. Though difficult, to pursue polypharmacology-based drug design, one needs to first select the biological target combinations to be modulated simultaneously. Target selection is a critical aspect in polypharmacologic drug design, and it is necessary to evaluate the targets over a few critical parameters.

The most important parameter for target selection is to evaluate the potential of the target to modify the disease condition (Fig. 5A). This requires a deep understanding of the target-disease association in terms of its disease pathway, and the effect brought about by modulating the target [10,13,102]. The chosen target also needs to be selective. This is especially important when dealing with infection and cancer where the target needs to be significantly overexpressed. Selectivity also implies that the targeted protein should not have a corresponding homologous protein in the host (organism that developed infection or cancer), or if present, the homologous protein should be significantly different from that in the targeted cell (Fig. 5A) [102].

One of the key arguments in favor of polypharmacologic drug design is that simultaneous modulation of multiple targets results in an additive or synergistic effect, resulting in a lower effective dose as well as fewer side effects (Fig. 5B). This provides a key parameter for target selection. For example, the dual histone deacetylase (HDAC) - EGFR inhibitor CUDC-101, synergistically exhibited roughly a 5-fold higher cytotoxicity in salivary mucoepidermoid carcinoma when compared to HDAC and EGFR monotherapies [103].

Additionally, one needs to choose protein targets which are 'druggable', meaning the protein target should have the potential to be modulated by a drug-like molecule (Fig. 5C). One way to evaluate this is by mapping the active site coordinates of the target with known drugs or drug-like compounds. Furthermore, a protein is more likely to be considered druggable if it belongs to a protein family where at least one of its members is targeted by a drug [102,104].

The choice of a target combination to be simultaneously modulated also depends on various parameters. When a protein is targeted, it is the signaling pathway which is intended to be modulated and not the isolated protein [102]. Furthermore, it has been shown that when several targets are partially inhibited, it results in an effect which is greater than complete inhibition of just one of those targets [105]. Based on this premise, selecting a target combination from different signaling pathways (parallel targeting in network pharmacology) would be of particular importance if it is intended to prevent compensatory homeostatic responses or adaptive resistance mechanisms, such as in infection and cancer. Csermely et al. demonstrated that multi-targeting antimicrobial agents were much more effective when their targets spanned across an entire network, rather than a single target [102,105,106]. Vertical targeting is another method in network pharmacology, where different nodes of the same signaling pathways are targeted. This method is useful in target mutation type resistance, where one of the protein targets itself gets mutated [102,107]. Target combinations which form highly connected nodes in a signaling network would be an ideal strategy if the intention is to eliminate pathogens or cancerous cells. On the other hand, if the treatment objective is to repair a dysregulated network to a healthy state, selecting multiple non-crucial target nodes may be advantageous in order to avoid the side-effects associated with targeting the key network nodes [83,102]. However, small molecules with reasonable multi-targeting activity could have low selectivity for the therapeutic targets as compared to other closely related antitargets, which could possibly lead to side effects. Nevertheless, this problem could be tackled if the simultaneous activity at the different therapeutic targets demonstrated significant synergy. This would allow for the lowering of the therapeutic dose such that there is no appreciable activation of the antitargets. Such a synergy would also prevent the side

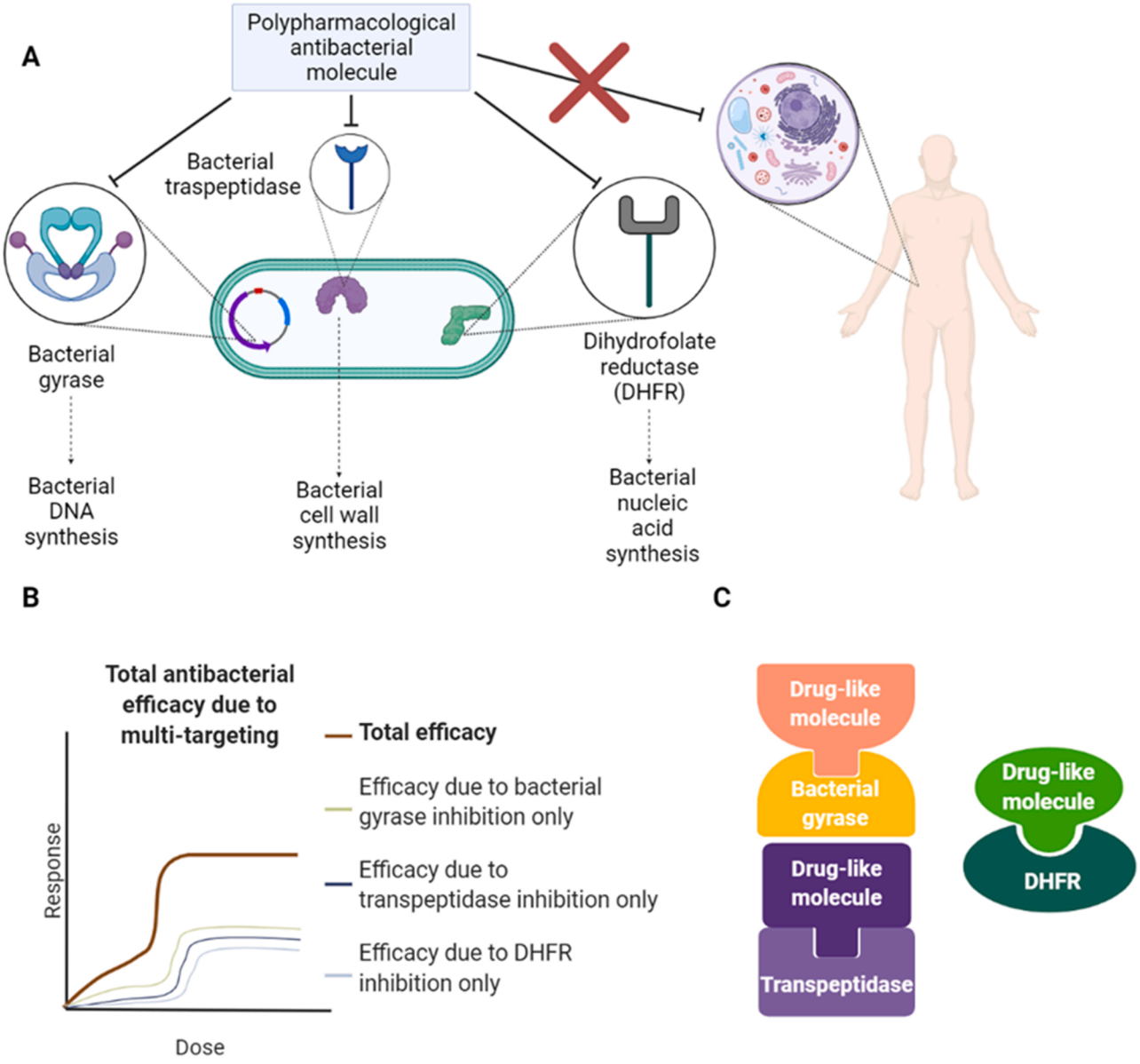

Fig. 5. Target selection for antibacterial therapy. A) The targets should have the potential to modify the disease state (cause bacterial cell death) and should be exclusive in bacteria. B) Simultaneous target modulation should result in additive or synergistic effect. C) The targets should have the potential to be modulated by a drug-like molecule. Created with Biorender.

effects associated with high potency single-targeting agents. Furthermore, such synergistic activity on therapeutic targets is beneficial when the synergy is exclusively observed in the disease pathway, despite the individual targets being present in healthy tissue. This lack of target synergy in healthy tissues would result in alleviated drug side effects [105].

There are a few valuable online resources, such as therapeutic target database and multiple target ligand database (e.g., ChEMBL, Drug target commons, and BindingDB), which aid in selecting the right target combinations for polypharmacology [108–111]. Despite this, the overall process of target selection is difficult. Once the target combinations are finalized, one needs to incorporate the pharmacophores of drugs or drug-like candidates which act on those targets [10]. There are a few methods to achieve this, each having its advantages and limitations.

3.3.2. *Multi-targeting ligand design*

Chemically, the key aspect that defines a multi-targeting molecule is the pharmacophoric features of the multiple targets it modulates simultaneously. There are three general methods by which one can amalgamate the multiple pharmacophoric features.

3.3.2.1. Linked pharmacophores. A seemingly straightforward method of combining multiple pharmacophores is by simply conjugating them via a chemical linker. In this approach, the linking group is critical to the behavior of the newly formed molecule [7,112,113]. Not only would the linker affect the way the molecule interacts with its targets, but also affect its overall pharmacokinetics. For example, a suitable linker can impart polarity to the molecule and in turn improve its solubility [7, 114].

In the case of a cleavable linking group, the linker chemistry would determine if, where, and how the linked molecules would be separated from each other (e.g. via pH change or enzymatic cleavage) [7,113]. Cleavable linking groups such as hydrazones, oximes, and thiomaleimides are acid labile, while disulfide linking groups are cleaved by reducing agents [7,115]. Linking groups such as β-glucuronic acid and imide groups would be prone to enzymatic cleavage by β-glucuronidase

and endopeptidase, respectively [7,116,117]. Once the labile group in the linker is cleaved, modern linkers would undergo intramolecular reactions leading to the complete release of the linker and spacer groups from the linked pharmacophores [7,118].

For non-cleavable linkers, not only is the linker length and geometry important, but also the point of attachment to the pharmacophore is crucial [7,119]. This point of attachment is important for retaining the affinity of the linked pharmacophores for its respective biological targets. Additionally, the structure-activity relationship (SAR) of the linked pharmacophores would help in determining the suitable positions for linker attachment [7]. Therefore, choosing the appropriate linker type and point of attachment to the pharmacophores is key to this approach.

The concept of linked pharmacophores is interesting, especially in designing antibody-drug conjugates. Here, one of the two linked pharmacophores (antibody) aims at being the targeting agent, which delivers the second pharmacophore (a small molecule intended to modulate the target) to its intended site of action. This concept of antibody-drug conjugates has now been increasingly replicated in small molecule-drug conjugates, where a small molecule acts as a targeting agent. It has found great utility in anticancer and antimicrobial therapies, especially when the treating molecule is too cytotoxic to be given as a standalone monotherapy [7,113].

Similar to the antibody-drug conjugate system discussed above, proteolysis-targeting chimeras (PROTACs) is a relatively new and promising approach. PROTACs are dual acting molecules which activate the ubiquitin-proteasome system (UPS) to degrade a disease related protein. They consist of two components connected via a linker: a ligand that binds to the E3 ligase, and another ligand which binds the desired protein to be degraded. The rationale behind such a dual component system is that the protein to be degraded would be brought in close proximity to the E3 ligase, thereby facilitating its proteasomal degradation, which otherwise would have been difficult or not possible at all [120]. In 2008, Crew et al. synthesized PROTACs consisting of nutlins linked to a non-steroidal androgen receptor ligand (SARM). Nutlins are known binders of the E3 ligase murine double minute 2 (MDM2). This results in the recruitment of the androgen receptor to MDM2, facilitating its ubiquitination and subsequent proteasomal degradation, ultimately providing a potent therapy for prostate cancer [120–122]. One of the first examples of linked pharmacophores designed with the intention of targeted chemotherapy to cancer cells was reported in 1992, where cytotoxic drugs were linked to cholic acid derivatives resulting in their uptake into the targeted cells via bile acid transporters [7,123,124]. Since then, many multi-targeting agents have been linked to target cell populations via specific transport systems and cell surface receptors, such as estrogen receptors and glucose transporters [7,113]. It is important to note here that while drug-antibody conjugates and PROTACs are linker-based entities that improve overall efficacy by improved targeting, these molecular entities would not clearly fall under polypharmacology. For them to be polypharmacologic, they would need to simultaneously modulate multiple targets which are involved in a disease, such that the concurrent modulation would result in additive or synergistic efficacy.

Other than aiding in targeted drug delivery, linking pharmacophores to exhibit superior pharmacodynamic effects on the multiple targets via synergistic activity has also increased in prominence, especially in the antimicrobial field [7,125]. In one study, with the aim to expand the spectrum of activity to both gram positive and negative bacteria, Plech et al. linked ciprofloxacin to the 1,2,4-triazole system - a pharmacophore known to be effective against gram positive bacteria only (Fig. 6A). A total of 18 hybrids were synthesized and their antibacterial activity was evaluated against gram positive *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Bacillus cereus*, *Micrococcus luteus* and gram negative bacteria *Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa*. A methicillin resistant strain of *S. aureus* was also used. Out of the 18 hybrids, 11 showed enhanced antibacterial activity in both gram positive and negative bacteria when compared to ciprofloxacin alone, with the best result being 8.5-fold more potent against *S. aureus* [126,127]. Through this study, Plech et al. showed that by linking pharmacophores, superior pharmacodynamic effect could be achieved by simultaneously targeting gram positive and negative bacteria. However, it was not explored if these hybrids effectively combined their separate mechanisms of action to achieve their superior antibacterial activity.

Despite its advantage of modulating multiple targets via a single molecule [7,113], this approach also typically results in molecules with large structures which may fail to provide the necessary bioavailability or may be too large to cross biological membranes and reach the site of action [7,128,129]. Additionally, if the linker design and position is incorrect, it would hinder in the pharmacophores' binding to its target. In any case, the design and attachment of the linker is of prime importance in this approach [7].

3.3.2.2. Fused pharmacophores. In addition to using a chemical linker, pharmacophores can be combined into a single molecule by simply fusing them. In this approach, the frameworks are directly covalently linked. This approach is most possible when the pharmacophores have a certain degree of overlap in their structures [128]. The characteristics of the fused multi-targeting compounds can be cleavable or non-cleavable, depending on the nature of bond linkage between the two pharmacophores. For example, if the pharmacophores are fused together by an

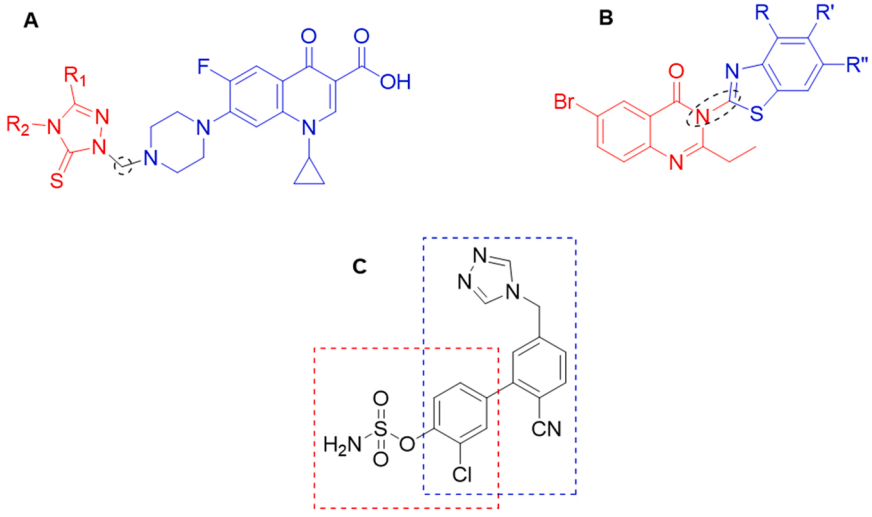

Fig. 6. There are 3 main methods to design a multi-targeting drug. A) By linking pharmacophores. Linking ciprofloxacin (blue) to the 1,2,4-triazole system (red) via a one-carbon linker (dotted circled). B) By fusing pharmacophores. Molecular fusion of quinazoli-4(3 H)-one (red) and 2-amino benzothiazole (blue) pharmacophores. The dotted circle indicates the point of fusion. C) By merging pharmacophores. Merged and optimized anti-aromatase (blue dotted box) and anti-steroid sulfatase (red dotted box) pharmacophores.

ester, an imine, or a hydrazone moiety, it can be cleaved in vivo [7,118].

Similar to linked pharmacophores, fused pharmacophores require the site and method of fusion to be optimized. This is especially true in cases of non-cleavable fused pharmacophores, where the fusion of the pharmacophores might hinder the compound's ability to bind its targets due to masking of the key binding functional groups [7].

This strategy has been shown to be successful in designing fused multi-targeting compounds for anticonvulsant activity. Currently available anticonvulsants are effective, however, they do present side effects such as hepatotoxicity [130,131]. In order to design anticonvulsant agents with little to no hepatotoxicity and neurotoxicity, Ugale et al. fused two anticonvulsant pharmacophores: quinazolin-4(3 H)-one and 2-amino benzothiazoles (Fig. 6B). The resultant derivatives possessed anticonvulsant activity and did not exhibit any signs of neurotoxicity or hepatotoxicity [131].

Fusing two pharmacophores without a chemical linker allows for the design of a compact multi-targeting compound that is not as bulky as the linked pharmacophores, and potentially has a better pharmacokinetic profile. However, chemical fusion of two pharmacophores and subsequent optimization, while maintaining activity, is a very difficult task [7,132]. In addition to the methods discussed previously, a third method of designing a multi-targeting molecule which results in compact molecules, is accomplished by merging pharmacophores.

3.3.2.3. Merged pharmacophores. The best drug-like multi-targeting molecules can be designed by merging the pharmacophores [7,128,133,134]. Essentially, the key pharmacophoric features required to interact with the targets are amalgamated into a single molecule. If the different types of multi-targeting molecules were to be classified based on the degree of pharmacophoric mergers, linked pharmacophores would be at one end of the spectrum, and merged pharmacophores would be at the other end, where the pharmacophores are now integrated into a single molecule [128]. For such a merger to be possible, there needs to be a certain degree of structural overlap in the pharmacophores of the individual targets [7,134]. Another requirement would be that the binding sites of all the targets should be capable of accommodating the same chemical moiety (e.g., geometry and charge distribution). Pharmacophoric features should also be compatible with the other target [7,135].

To design a merged multi-targeting ligand, identifying the lead compound as a starting point for optimization is of key importance. With knowledge of known ligands for specific targets, common structural and pharmacophoric features can be methodically merged into a minimal and common lead pharmacophore. Additionally, x-ray crystallographic information is of great value in identifying these key structural features which are crucial for activity [7]. Woo et al. designed merged multi-targeting compounds by combining the pharmacophores for aromatase inhibition and steroid sulfatase (STS) inhibition (Fig. 6C). Using information from X-ray crystallographic and docking studies, they designed a dual inhibitor with nanomolar potency (aromatase IC_{50} = 100 nM, STS IC_{50} = 227 nM). They incorporated the crucial phenol sulfamate moiety, which is necessary for irreversible steroid sulfatase inhibition, into a known aromatase inhibitor scaffold. With extensive SAR studies and structure optimization, their dual inhibitors then reached a potency of picomolar value (aromatase IC_{50} = 500 pM, STS IC_{50} = 5.50 nM) [7,136–139].

In addition to using a rational approach of merging common structural and pharmacophoric features, several computational approaches can also be used to design merged multi-targeting agents. Publicly available databases, such as ChEMBL and PubChem BioAssay, can be used to build a network of drug-target pairs and discover chemical scaffolds which can be utilized for merged multiple ligand development [7,140,141]. PubChem BioAssay is the main database for in vitro screening data, while ChEMBL is for bioactivity data curated from the scientific literature [134]. For such approaches, molecular descriptors of the molecules are required which can then be compared for similarity using molecular fingerprinting techniques [7,134]. Molecular fingerprinting is a computational technique in which structural characteristics of a molecule are encoded as a vector [142]. Numerous fingerprinting techniques exist which can compare the molecular structures for similarity [134,143]. In addition to these methods, previously discussed structure- and ligand-based computational methods can also be used in designing merged pharmacophores [7].

The methods described above greatly aid in the *de novo* merging of multiple pharmacophores. Besides these, another intuitive way of designing merged pharmacophores would be to begin with a molecule that is known to possess activity against one of the targets, and then stepwise adapt that molecule with additional structural elements required for activity against the complementary target [7,128]. The challenge of this approach lies in strategically adding structural features such that affinity to the complementary target is enhanced, while retaining the affinity to the original target. However, this challenge can be met when the two targets have similar overlapping pharmacophoric requirements and if the parent compound has at least some minimal activity at the complementary target [128]. Furthermore, a systematic SAR of the added substituents should be performed to check the preservation of activity against both targets [6,144]. The advantage of this design-in approach is that sufficient SAR information is already available for the two to-be merged compounds, and this can be used for optimizing the merged compound [7].

On the flip side of the design-in approach is the design-out approach. Instead of merging the pharmacophores of two selective compounds by structural adaptation, the design-out approach generally begins with a compound that modulates not only the multiple targets of interest, but also the antitargets [7,144]. The design-out approach aims at strategically removing structural features responsible for activity at the antitargets, thereby improving the selectivity to the targets of interest [144,145]. By analyzing the X-ray co-crystallized structure, the binding mode of the compound with the desired targets and antitargets can be compared, leading to the strategic disruption of activity at the antitargets. The design-out approach is typically less common because it is rare to find a lead compound possessing activity on multiple targets. However, the approach is still useful in cases where the number of targets exceeds two. This occurs due to each additional target exponentially increasing the challenges of using the design-in approach [7,144].

In general, regardless of the technique used, once a multi-targeting lead compound has been identified, a rigorous structural optimization process would follow. This will generate a high degree of potency against the multiple molecular targets and provide a balanced activity profile for the targets of interest. The challenge in achieving high affinity for all desired targets is that this often leads to molecules with a high degree of lipophilicity, as well as large structures with poor pharmacokinetics. Therefore, to allow for structural variation and enlargement, merged lead pharmacophores should be low molecular weight and focus on retaining low lipophilicity while also avoiding extensive structural enlargement [7,128,129,134]. Another objective would be to optimize the potency ratio of the multi-targeting ligand against its targets. To achieve this, one can start by delineating the molecule's activity against each isolated individual protein target and then determine the optimal balance in potency against the targets of interest. Ideally, the potency for each individual target should not differ by more than one order of magnitude [146]. Furthermore, to strengthen the proof of concept and evaluate the molecule's activity in the presence of various molecular pathways, cell-based assays and animal disease models should be used [7,128,146]. The results of these experiments should show better activity with the multi-targeting molecule than the single-targeting reference molecules used either alone and in combination [146].

The optimization process can make use of structural information, computational methods, and information from molecular docking. For example, the molecule zafirlukast was identified to have triple activity comprising of cysteinyl leukotriene 1 receptor (CysLT1R) antagonism, peroxisome proliferator-activated receptor- γ (PPARγ) agonism, and

soluble epoxide hydrolase (sEH) inhibition in vitro, but lacked an activity profile balance. The activity profile balance was then optimized purely using the molecular docking results. A close study of the compound's proposed binding modes for PPARγ and sEH, led to very minor structural changes resulting in a sufficient increase in PPARγ and sEH potencies, ultimately balancing the triple modulator's activity profile [7,147].

With the methods and techniques discussed, one can predict and intentionally incorporate promiscuity into their molecules. In addition to enhancing potency and efficacy in treating complex diseases, promiscuity and therefore polypharmacology, have numerous other applications.

4. Applications of polypharmacology

4.1. Drug repurposing

One of the most useful applications of polypharmacology is in drug repurposing [148], which refers to the use of an already marketed drug for a new indication [12,149,150]. Recently, it has gained more interest as many approved drugs have been evaluated in clinical trials for new indications [148,151,152]. This is advantageous as developing an already established drug for a new indication is typically faster, cheaper, and less risky than developing a novel drug. Additionally, an approved drug's redevelopment for a new indication is facilitated by previously established studies, such as pharmacokinetic, toxicity and formulation studies in humans [12]. Molecules which are not approved or have been withdrawn from the market due to adverse reactions can possibly be 'rescued' if the benefits of their repurposed indication justifies their administration [153]. A classic example is the revival of thalidomide after its withdrawal in 1961 due to its severe teratogenic effects. In 1964, a serendipitous discovery led to the repurposing of thalidomide to treat erythema nodosum leprosum (ENL), a very painful inflammatory complication of leprosy. This repurposed indication is a result of thalidomide's polypharmacologic activity on tumor necrosis factor α (TNF-α), which is abnormally produced in inflamed tissue. Currently, thalidomide is used for the treatment of multiple myeloma, and its efficacy is due to its multi-target activity on interleukin-6 (IL-6) and VEGF [12,154,155].

Drug repurposing also includes repurposing a marketed drug that has a therapeutically useful side effect. For example, codeine has a known side effect of causing sleepiness. This side effect could in fact be useful in patients who struggle with falling asleep. Such repurposing alters the affinity profile of a promiscuous compound from one therapeutic target to another. For example, sulfacarbamide was a popular sulfonamide antibacterial with a short half-life which was derivatized into the longer acting derivative **1**. However, derivative **1** presented a prominent side effect of hypoglycemia. The drug was later derivatized into carbutamide and marketed in Europe as an oral antidiabetic drug. However, carbutamide exhibited residual antibiotic activity, which raised concerns of it developing antimicrobial resistance. Though carbutamide was not approved in the U.S. due its unacceptable side-effects, it was further derivatized into tolbutamide, which acted on adenosine-5'-triphosphate-sensitive potassium channels (K_{ATP} channel) to become an important treatment for noninsulin-dependent diabetes, while also being devoid of antibiotic activity [12,156,157].

Drug repurposing is widely prevalent in the development of anticancer therapeutics as well. In fact, numerous collaborative initiatives have been aimed at drug repurposing to develop new treatments. For example, Repurposing Drugs in Oncology (ReDO) is an international collaborative effort with the purpose of bringing less toxic cancer treatments to patients in a shorter period of time [148,158]. It offers a database of drugs approved for indications other than cancer but have shown some anticancer activity in preclinical and clinical settings, demonstrating the potential to be repurposed. Another such initiative is Repurposing, Focused Rescue, and Accelerated Medchem database (ReFRAMEdb), which provides a collection of ~14,000 drugs and their bioactivity data which are either approved by the Food and Drug Administration (FDA) or are currently experimental drugs.

During the current COVID-19 pandemic, drug repurposing has been one of the leading strategies utilized to develop an effective cure in a short time frame [159]. The NIH have awarded millions of dollars in funding with the goal of utilizing clinical trial data of existing drugs in an effort to identify utility in repurposing [160]. Using the computational methods previously discussed, Jang et al. discovered emodin, omipalisib, and tipifarnib to have anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) activity in human lung cells. Furthermore, upon testing three drug combinations, omipalisib-remdesivir, tipifarnib-omipalisib, and tipifarnib-remdesivir, all exhibited strong synergistic effects in inhibiting SARS-CoV-2 [159]. These examples highlight the importance of drug repurposing as a prominent strategy based on polypharmacology.

4.2. Polypharmacology in epigenetics

Epigenetics is the study of the heritable and reversible changes of gene expression that occur due to changes other than that in the deoxyribonucleic acid (DNA) sequence itself [161,162]. The first epigenetic modulators to be approved by the FDA were azacytidine (2004) and decitabine (2006). These drugs inhibit DNA methyltransferase (DNMT) enzymes and were approved for the treatment of hematological malignancies. Recently, clinical candidates for other epigenetic targets, such as lysine methyltransferases (KMTs), arginine methyltransferases (PRMTs), lysine demethylases (KDM), and bromodomains (BRDs), have also been developed. Despite many of these drug candidates having a variety of epigenetic targets, one of the reasons why a single epigenetic-targeting approach could be ineffective is due to the development of pharmacological resistance. In cancer, phenotypic abnormalities occur mainly due to signaling pathways evolving over time to circumvent drug-induced effects of growth inhibition, cell death, DNA repair, and metabolic alterations. The key to designing a successful epigenetic targeting drug would in fact be a multi-targeting drug which acts on multiple nodes of the signaling pathway [161]. In such epigenetic polypharmacology, the multi-targeting drug could simultaneously modulate either an epigenetic target along with a non-epigenetic target, or two epigenetic targets simultaneously. Targeting different pathways at once can greatly decrease the chances of acquired resistance due to one of the targets compensating for the other. Most multi-targeting epigenetic drugs developed thus far have comprised of a histone deacetylase inhibitor (HDACi) linked to another pharmacophore (typically a tyrosine kinase inhibitor, or TKi)[148,163,164]. The development of such a multi-targeting epigenetic drug stems from the fact that coadministration of HDACi and TKi have shown synergistic effects in vitro and in vivo models [161,165,166]. One such HDACi/TKi multi-targeting molecule, CUDC-101, is currently in phase 1 clinical trials for cancer patients [161].

In general, the multi-targeting nature of a polypharmacologic molecule is greatly applicable to the treatment of complex disorders such as cancer, CNS disorders, and infection. Targeting multiple nodes in these disease pathways often results in much greater efficacy, resulting in lowered effective doses, which in turn reduces toxic side effects [7,103]. Targeting multiple pathways at once also increases the potential for overcoming, or at least delaying, drug resistance, which is a major reason for drug ineffectiveness [21,22].

5. Summary and outlook

Conventional research has aimed at designing highly specific molecules to avoid adverse effects due to antitarget binding. This approach has been successful in delivering single-targeting drugs, however, to treat a complex and multi-factorial diseases, a single target approach has not been as efficacious [4,5,7,12]. Retrospective analyses of a few

successful drugs revealed them to be multi-targeting, indicating a possible link between their therapeutic efficacy and their multi-targeting nature [11]. These findings have suggested that the sub-efficacy of certain drugs is due to their single-targeting nature, and so polypharmacology has been identified as the next paradigm in drug discovery [13].

By simultaneously modulating multiple targets, polypharmacologic drugs not only provide superior efficacy, but also help prevent or delay drug resistance; which is a major source of ineffectiveness for anticancer and antimicrobial drugs [7]. Despite this, pharmaceutical industries are reluctant to incorporate polypharmacology into non-complex disease drug research since the process of lead-to-drug optimization is much more complex for multi-targeting compounds. This is due to difficulty in retaining drug-like properties while balancing activity against multiple targets. Additionally, interactions with antitargets often cannot be avoided, causing safety concerns. The translatability of animal disease models to humans is also not very reliable [12]. Finally, the optimal ratio of pharmacological activities on different targets may not be necessarily be 1:1 and is difficult to elucidate in the early stages of drug development [12,145].

With current technology, however, there is improved elucidation of the molecular mechanisms and the multiple underlying factors involved in diseases. In particular, advancements in computational methods, such as signaling network analysis and quantitative flux modeling, have made it easier to accurately select a set of targets; simultaneous modulation of which, would result in synergistic effects [7]. Computational methods such as pharmacophore modeling, binding site analysis and comparison, and similarity ensemble approach combine biological and structural databases aiding in accurately predicting promiscuity and antitarget interactions. These methods have played an instrumental role in the current growth and success in multi-targeting drug design. They provide critical data regarding a molecule's potential to be polypharmacological long before it is synthesized, ultimately saving time, money, and effort. The result of these iterations also provides key information for the structural optimization of the hits. These computational methods when combined with in vitro and in vivo experimental approaches provides more accurate data in identifying synergistic target combinations and in confirming promiscuity.

In summary, we feel designing a polypharmacological drug is an uphill battle and is more difficult compared to designing a single-targeting drug. However, with the current technology and information available regarding different diseases and chemical functional groups, it has become more plausible to intentionally design a polypharmacological drug. Through this review, we aimed to provide a substantial argument advocating for the need of polypharmacology in fulfilling the shortcomings of the current therapies. At the same time, we contrast the potential difficulties one would encounter while pursuing polypharmacological drug designing. We speculate this review to be a guide, delineating the advanced tools, databases, methods, and red flags which one would need along their journey in pursuit of a polypharmacologic lead.

CRediT authorship contribution statement

Abbas Kabir: Conceptualization, Writing – original draft. **Aaron Muth:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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