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We report on fragment-based and computational approaches as possible ways to accelerate and optimize the discovery of multitarget drugs.

The role of fragment-based and computational methods in polypharmacology

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Polypharmacology-based strategies are gaining increased attention as a novel approach to obtaining potentially innovative medicines for multifactorial diseases. However, some within the pharmaceutical community have resisted these strategies because they can be resource-hungry in the early stages of the drug discovery process. Here, we report on fragment-based and computational methods that might accelerate and optimize the discovery of multitarget drugs. In particular, we illustrate that fragment-based approaches can be particularly suited for polypharmacology, owing to the inherent promiscuous nature of fragments. In parallel, we explain how computer-assisted protocols can provide invaluable insights into how to unveil compounds theoretically able to bind to more than one protein. Furthermore, several pragmatic aspects related to the use of these approaches are covered, thus offering the reader practical insights on multitarget-oriented drug discovery projects.

Introduction

Complex diseases and polypharmacology

In recent years, the dominant paradigm in drug discovery has been the design of maximally selective compounds ('magic bullets') that target a single biomolecule thought to be individually responsible for a certain disease [1]. This target-centric approach has been very successful for diseases with a clearly defined mechanism, etiology and pathophysiology. However, there is a plethora of diseases with more-complex pathological mechanisms, for which the classic 'one target, one drug' paradigm has partially or fully failed [2]. In this scenario, drugs acting on multiple targets (the so-called multitarget drugs or 'magic shotguns' [1,3]) could offer superior efficacy profiles compared with single-target drugs. This is because they can better tackle the complexity of multifactorial diseases [4]. The multitarget approach has been proposed for central nervous system disorders, where genetic, biochemical and environmental factors can play a part in disease development [5]. In particular, researchers have found that several clinically effective

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drugs for depression [6,7] and schizophrenia [2] are pharmacologically complex and exhibit pleiotropic actions. This opens up new polypharmacological avenues for discovering innovative and effective therapies [8]. Neurodegenerative diseases, like Alzheimer's [9], Parkinson's [10], among others, also show rather complex etiopathologies. Here too, multitarget drugs could lead to novel and more-effective medicines [11]. Multitarget approaches have also been proposed as crucial in the search for novel therapies against cancer and infectious diseases [12–16], where the system complexity can arise from the resistance of either the cancer or parasitic cells [12]. Drug resistance is usually triggered by the appearance of one or more mutations in the genetic encoding for drug target proteins. The probability of a cell developing resistance simultaneously to multitarget drugs acting on unrelated proteins is statistically lower than the probability of resistance developing against single-target drugs. Therefore, chemotherapy-induced drug resistance could potentially be overcome by using multitarget drugs [17].

In the past, promiscuity has been seen as one of the major limitations of novel drugs because of potential side effects. However, a selective polypharmacological profile of a new chemical entity could provide drug candidates with a superior efficacy profile. In addition, compared with drug combinations (see below), multitarget compounds could also show superior pharmacokinetic (PK) and safety profiles. This all points toward polypharmacology being one of the most promising and innovative paradigms in the search for new drugs to treat complex diseases.

Multitarget drugs and combination therapy

A logical alternative to multitarget drugs is combination therapy; that is, using different drugs with different mechanisms of action to cure complex diseases. This practice is well established in anticancer chemotherapy and in the field of infectious diseases [18]. Combination therapy is also used to treat central nervous system disorders. For instance, the standard treatment of Alzheimer's disease is the combination of an acetylcholinesterase inhibitor with memantine [19], the only *N*-methyl D-aspartatic acid (NMDA) receptor antagonist marketed as an anti-Alzheimer's disease drug.

But the use of drugs that have multiple biological properties could have inherent advantages over combination therapies. It would obviate the challenge of coping with multiple drug entities, which could have different bioavailabilities, PKs and metabolisms. Administering one compound with multiple biological actions guarantees the simultaneous presence of the molecule in those districts of the body, where the active principle needs to work and interact with its multiple targets. Moreover, in terms of PK and ADMET optimization, the (pre)clinical development of a drug that can hit multiple targets should not, in principle, be different from the development of any other single lead molecule. It therefore offers a far simpler approach than the development of new combination therapies. In addition, the risk of possible drug–drug interactions would be avoided and the therapeutic regimen greatly simplified. Compliance with prescribed medication regimens is essential for effective treatment, but is particularly challenging for age-related diseases [20]. In light of these considerations, the development of multitarget agents could offer an efficient and cost-effective alternative to drug combinations.

Designing multitarget drugs

Although it is still in its infancy, the field of multitarget drugs has been booming over the past five years, with new agents coming to the market at a rapid pace, especially in the fields of oncology [15] and depression [6]. Moreover, many drugs with a multitarget profile are in clinical use today. However, their modes of action have usually been discovered only retrospectively.

The rational design of multitarget ligands with predefined biological profiles can be exceedingly challenging. This is because researchers must deal with the crucial issue of affinity balance toward different target proteins. This can be a resource-hungry step in the early discovery phase. In addition, the right balancing of target occupancy for achieving the desired *in vivo* efficacy profile is a further key challenge in multitarget drug discovery. Largely because of these aspects, the multitarget approach continues to meet stiff resistance from some within the drug discovery community. With this in mind, one possible way of limiting the costs associated with multitarget strategies is to design dual- rather than multitarget compounds. These could still show a superior efficacy profile to single-target drugs, but would be more feasible than multitarget compounds in terms of affinity balancing and *in vivo* profiling. Fig. 1 summarizes a possible dual-target design strategy, as also suggested by Morphy and Rankovic [4]. First, researchers must carefully select two pharmacologically relevant targets (target A and target B) located on complementary pathological pathways. The selection should be based on chemical and pharmacological considerations. From the start, researchers must question whether or not modulating the two selected targets could lead to additive effects or synergistic potentiation (Box 1). Then, the pharmacophoric functions responsible for binding to targets A and B must be identified. Finally, the key pharmacophoric functions can be amalgamated in one dual-target compound to obtain

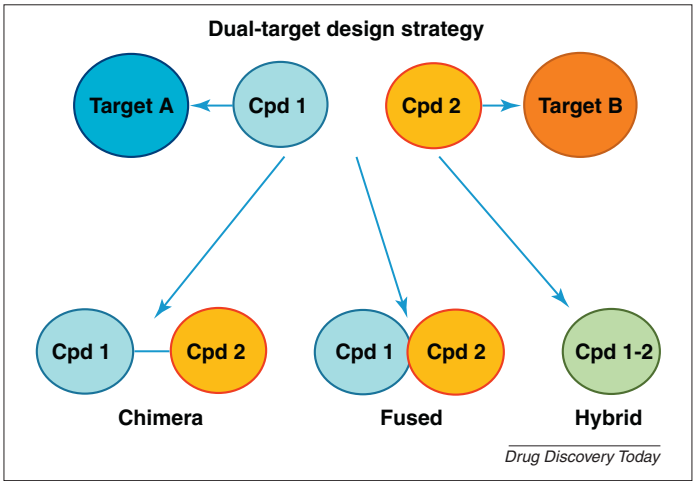

FIGURE 1

Dual-target design strategy. A possible way of designing dual-target drugs is to develop new chemical entities that can modulate the biological activity of two targets belonging to complementary pathways. This is likely to lead to synergistic potentiation (Box 1). For example, by combining Cpd 1, responsible for modulating target A, with Cpd 2, able to interfere with target B, one can design new dual-target compounds according to the following strategies: (i) linking, by means of suitable spacers, of the key pharmacophoric functions (chimera); (ii) fusion of the key functions (fused); (iii) amalgamation of the essential pharmacophoric groups into one molecule (hybrid).

hybrid, fused or chimeric compounds. The decision to generate hybrid, fused or chimeric compounds will be driven by the nature of the targets, the availability of reference compounds and the chemical feasibility. From this schematic of the design process it clearly emerges that innovative technologies, like fragment-based and computational methods, can play a crucial part in multitarget drug discovery. As mentioned before, polypharmacological drug discovery can be very expensive, so innovative technologies are particularly important in helping to cut the high costs of designing, discovering and, eventually, developing multitarget drugs.

In this article we will review the major advances in the use of fragment-based and computational approaches to enhance and facilitate the multitarget drug discovery process. We will then provide some perspectives on a potentially wider application of these approaches in the field of polypharmacology and complex diseases.

We are confident that this review will help those readers newly entered into the polypharmacology field to appreciate better how innovative technologies can be applied to multitarget drug discovery.

Fragment-based approaches to multitarget drugs

In the mid-1990s researchers developed the technology to detect inhibitory activity sensitively in the μM–mM range. This opened up the possibility of designing drugs by step-wise addition of functional groups to simpler low-molecular-weight chemical entities (Box 2) [21]. Since then, fragment-based approaches have played an increasingly important part in academia and industry alike [22]. Today, they are well-established drug discovery tools for identifying small, highly efficient molecules as initial starting points [23]. Moreover, as the search for 'magic shotguns' slowly gains a wider following, so too will fragment-based approaches, which, by design, are more probable than conventional campaigns to unveil multitarget hits [24].

From promiscuity to multitarget opportunity

As early as 1988, Evans and colleagues reported the existence of chemical scaffolds with a higher propensity for binding to different proteins [25]. The authors argued that such promiscuous scaffolds should be judiciously modified in the search for new and highly selective modulators. What is apparent today is the fact that this attribute could be worth exploiting in the search for multitarget drugs. Interestingly, when target selectivity was the main goal, the propensity of some scaffolds to bind promiscuously was an inconvenient feature, which had to be overcome while growing the molecule. As early as a decade ago, Hajduk and coworkers tested, using NMR-based techniques, the binding preferences of several protein targets using a set of 104 fragments, which were derived from the deconstruction of bigger molecules [26]. The study showed that some privileged scaffolds have a high propensity for binding to proteins. Hence, the authors commented that identifying such scaffolds could help in designing screening libraries enriched with molecules containing those fragments. This would increase the probability of developing potent (selective) and larger lead-like inhibitors. Later on, Hann and colleagues used a simplified model to calculate the probability of interaction between proteins and ligands of diverse complexity [27]. The final conclusion of the study was that smaller molecules were better starting points for drug discovery. This is because the lower the complexity of a molecule the higher its chances of hitting biological targets. Several years later, Hopkins and coworkers finely analyzed the same concept within the context of the rational design of multitarget ligands [28]. In this case, information about the binding promiscuity of compounds was extracted from Pfizer corporate screening data on a statistically relevant number of diverse biological targets (220) and compounds (75 000). The authors found an inverse correlation between mean molecular weight (MW) and promiscuity (given a threshold activity of

BOX 1

Synergistic potentiation.

Multitarget drugs have therapeutic advantages over single-target drugs because they can show either additive or synergistic effects. An additive effect is when the simultaneous modulation of two targets by the same molecule is equal to the sum of the activities on each target alone produced by reference selective compounds. Synergy occurs when the overall effect is superior to the sum of the single activities. In this context, target selection is crucial: (i) additive effects can be observed if targets are located on the same path; whereas (ii) synergistic potentiation can be achieved only if the selected targets are located on functionally complementary pathways. Both cases (additive and synergistic) require lower drug doses, and therefore a better safety profile can be expected.

BOX 2

Fragment-based drug discovery.

The aim of this Box is to introduce the reader to some aspects of fragment-based methods relevant for the main topic (i.e. polypharmacology) of the present review. For a more in depth description of fragment-based approaches to drug discovery the interested readers can refer to the following recent review articles [88,22].

According to the 'rule-of-three' fragments are usually defined as molecules with a molecular weight <300, H-bond acceptors and donors ≤3, and a log $P \leq 3$. In addition, rotatable bonds ≤3 and a polar surface area ≤60 can be used as extra parameters for their definition [89]. Being smaller than lead-like molecules, fragments usually bind weakly to targets with affinities ranging between 100 μM and 10 mM. Fragment-based drug discovery aims to find small binders of a desired target. These are then turned into bigger molecules by step-wise addition of functional groups or by direct joining. The rationale behind this process is that the overall ΔG of a drug's binding can be conveniently dissected into the contributions of its constitutive parts. Taking into account the entropy loss as a result of the molecule expansion, fragments usually have higher affinity:size ratios than their matching lead-like molecules. To this end, Hopkins, in 2004, introduced the concept of ligand efficiency (LE, defined as the free energy of interaction divided by the number of heavy atoms) as a ranking metric for small binders. This draws attention to fragments that use their constituting atoms in a more efficient way [90]. Fragment-based screening libraries can adequately cover large chemical spaces with a reduced number of entries, compared with HTS libraries, increasing the probability of finding hits [91]. Also interesting is the fact that fragments, being small, often have advantageous physicochemical properties from the outset.

10 μM), arguing that smaller molecules, having less negative interacting features, are more likely to establish interactions with multiple biological targets. More recently Chen and Shoichet used molecular docking and X-ray crystallography to work on several β-lactamases. They found that fragment molecules could show various degrees of promiscuity, at least for this class of enzyme [29]. Moreover, the authors noted that this behavior tended to fade with the progression of the inhibitors toward more-advanced phases, where the addition of chemical functionalities was attempted. Conversely, Chen and Hubbard's analysis of multiple screening campaigns from Vernalis did not highlight such a straightforward correlation between size and binding promiscuity [30]. In fact, only a very small percentage (i.e. 0.6%) of their dataset appeared to bind to multiple targets, whereas the majority of fragment hits showed high degrees of selectivity. Scientists at Vertex, in a more focused effort, analyzed the kinase-likeness, defined as the propensity of certain molecular motifs to bind protein kinases [31]. The analysis served to define rules for rapidly identifying molecules containing such privileged motifs to enrich screening libraries. Morphy's analysis of Organon's SCOPE database [32] emphasized a well-defined correlation between size and selectivity, supporting the hypothesis that the intrinsic simplicity of small compounds favors nonselective binding events [24]. Along the same lines, to shed light on the relationship between promiscuity and chemistry, Barelier et al. analyzed the outcome of NMR spectroscopy efforts to investigate the binding interactions between 150 fragments and five proteins [33]. Despite the limitations in size of their datasets, some important conclusions were drawn. For instance, low specificity was observed between homologous proteins or unrelated but poorly druggable proteins, whereas higher selectivity was achieved with highly druggable targets. Notably, in the cases of the latter two studies, the emphasis was placed not only on the fragments but also on the characteristics of the molecular targets, underlining how ligand promiscuity and the features of interacting partners are closely intertwined [34].

Possible strategies for fragment-based multitarget design

In light of the above considerations, fragment-based approaches are likely to detect promiscuous molecules. They are thus an attractive option for multitarget drug design. However, to date, little attention has been directed toward the potential of these methodologies as sources of initial hits [24]. This is probably because the attractiveness of polypharmacology has only recently started to gain momentum. However, we expect fragment-based methodologies to play an ever-increasing part in the multitarget field. Surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), NMR and X-ray crystallography methods can be used to detect weak binders with great success (Box 3) [22,35–37].

Screenings of small molecules against a panel of targets has the appealing potential to disclose areas of overlap, in the specificity landscapes, populated by scaffolds capable of modulating the activity of two or more biomolecules simultaneously (Fig. 2).

One advantage of using fragments instead of bigger molecules is the reduction in the available chemical space to search. However, it is still challenging to pinpoint hits within a crowd of possible candidates. In the earliest stages of the search for multitarget molecules, one crucial element is the design of fragment screening libraries [38]. The chemical space of the library should be diversified to ensure the presence of different chemotypes. Nevertheless, before compiling the library, it could also be convenient to extract information about target preferences from in-house-generated data, if available, or from publicly available databases such as ChEMBL [39], BindingDB [40] or WOMBAT [41], to name a few. In line with the findings of Bajorath and Hu in their recent retrospective study, this information could enrich the library with scaffolds containing fragments that are found to be well-accepted by the targets of interest [42]. Of course, it is still not easy to predict *a priori* whether two target proteins share an area of common binders at all. To address this, Miletti and Vulpetti developed a method based on pocket similarities and tested it on a panel of protein kinases and, subsequently, on proteins from the Worldwide Protein Data Bank [43]. Encouragingly, at least from a multitarget design standpoint, remarkable resemblances were found at the subpocket level, even between unrelated proteins. When looking for initial hits, medium-throughput techniques, such as ligand-observed NMR or SPR, should guarantee a good cost:benefit ratio. Here, the well-known risk of having false positives could be tackled by using an orthogonal validation (i.e. using different assay methods in parallel). This is particularly important where

BOX 3

Selected biophysical techniques used for fragment-based screening.

NMR methods can be divided roughly in two major categories: ligand- and protein-observed. Protein-observed methods detect changes in the NMR spectra of a protein upon ligand binding and can provide information about the mechanism of binding. The protein (which cannot exceed 40 kDa in size) must be available in large amounts in isotopically labeled form. The throughput is usually in the order of thousands of compounds.

Ligand-observed techniques, such as saturation transfer difference (STD) [92] and WaterLOGSY [93], monitor the changes in ligands' NMR spectra upon binding to a target protein. Because they are simpler to implement, such approaches are more widespread in drug discovery pipelines, especially at the early stages. There is no limitation in size for the protein and the throughput is usually higher than in protein-observed methods.

SPR spectroscopy provides kinetic and thermodynamic data of binding. Either the protein target or the ligand is attached to the surface of a sensor chip. The method relies on detecting the changes in the refractive index near the surface when a binding event occurs. The changes are proportional to the strength of the interaction. Although methods to enable massive screening via SPR have been developed, the throughput is usually in the order of thousands of compounds.

In X-ray crystallography for hit detection, cocktails of fragments are soaked into preformed crystals of the target protein. The analysis of the electron density (either manual or automated) allows the identification of the bound fragment. The throughput is not very high and no inhibition data can be inferred. False positives are not an issue; however, false negatives are.

ITC is not routinely used as a screening approach in fragment-based drug design, whereas it can play a part in further steps of the discovery process. ITC registers heats of association caused by protein–ligand binding. The free energy of binding is conveniently broken down in enthalpic and entropic contributions. The throughput is low and usually high amounts of protein are needed.

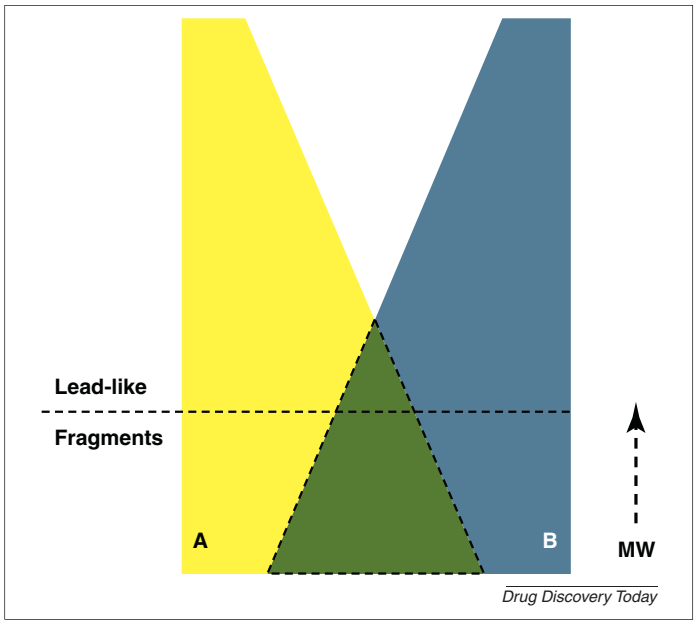

FIGURE 2

Overlap of the selectivity areas of protein targets A and B. The dotted triangle area is populated by compounds that can bind simultaneously to the two included targets. Dual-target activity is possible until a certain threshold of molecular weight (MW) is reached. This limit, along with the position of the boundary between fragments and lead-like compounds, is expected to be highly variable depending on the characteristics of the considered targets. For instance, in the case of kinases, even high-MW molecules can bind promiscuously. Nonetheless, in general at high MWs, compounds are likely to exhibit a more elevated level of specificity.

more than one target is involved at the same time. Recently, scientists at Astex Therapeutics showed that X-ray methods can also be used effectively in a relatively high-throughput manner as the primary method for hit detection [36]. Aside from the costs, another major drawback of using X-ray screening early on in the pipeline is that some binders could be missed because they are unsuitable for co-crystallization. The hit identification phase is a starting point that is usually superseded by several steps that aim to balance the activities toward the targets while making the molecule more drug-like by step-wise addition of functional groups. At this stage, along with NMR- or SPR-generated inhibition data, X-ray crystallography and protein-observed NMR could be invaluable tools in the multitarget hit-to-lead and lead optimization steps [44]. The experimental confidence about the position of the fragment at the protein sites could guide the chemist toward modifications that are predicted to be either beneficial for the activities toward both targets (in the case of two), or favorable for one and at least tolerated by the other. To this end, computational tools are expected to play an ever-increasing part in future because they are cheaper than experimental techniques. *In silico* prediction of the docking poses of relevant compounds could be used along with growing algorithms for hit-to-lead purposes [45]. As with ordinary single-target campaigns, the ligand efficiencies (LEs) toward the studied targets must be simultaneously monitored during the hit evolution, to pursue a defined activity ratio between targets. Ultimately, in the advanced stages, ITC assays could be used to dissect the enthalpic and entropic binding contributions, fine-tuning the optimization process.

Multiple solvent crystal structures (MSCS) in multitarget drug design

Since the mid-1990s X-ray crystallography has been successfully used to map protein hotspots [46]. The process, firstly suggested by Allen and colleagues and named multiple solvent crystal structure (MSCS), relies on the structural determination of a protein in the presence of varying concentrations of small organic molecules. Such molecules tend to displace water at precise locations thus highlighting possible binding sites of protein modulators [47,48]. However, to date, MSCS has been used only for single proteins, solvated with several different organic media, to design specific inhibitors by solvent molecule linking. Notably, researchers have found a direct correlation between the solvent concentration and the number of sites occupied [49]. This enables an accurate mapping of the strengths of association of solvent molecules to every available hotspot. In a fresh interpretation of the MSCS method, one could compare the proteins of interest, crystallized with the same set of solvents, and then look for similar patterns within the obtained mosaics of solvent molecules (Fig. 3). Such motifs could be used as starting points for rationally designing hits by step-wise addition of linkers and/or functional groups, thus obtaining chimeric or fused multitarget ligands. It is worth underlining here that, although strictly obeying the rule-of-three, the organic molecules used in

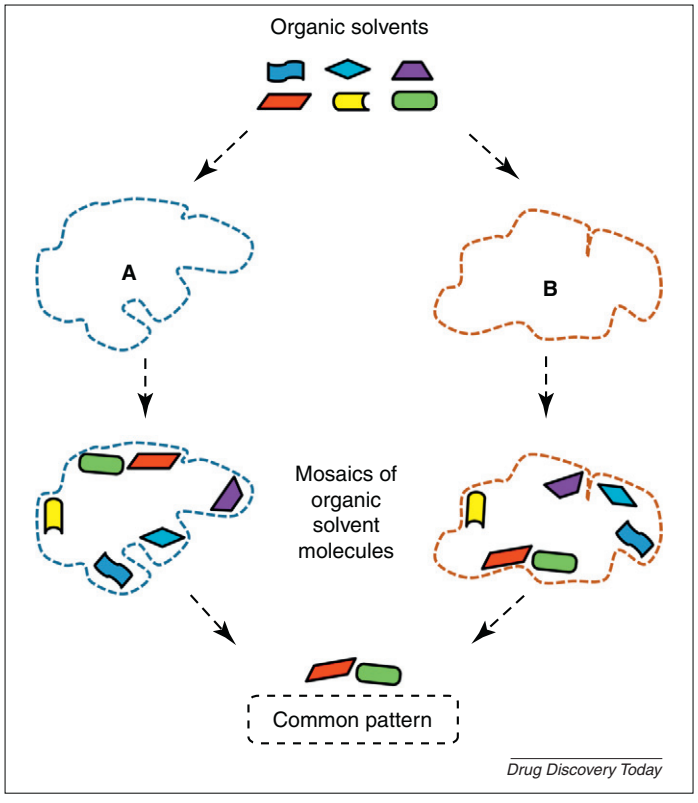

FIGURE 3

Multiple solvent crystal structure (MSCS) for multitarget drug discovery. The use of a set of organic solvents in X-ray crystallography could highlight common hotspots in two different target proteins (A and B). The colored shapes represent organic molecules binding at protein sites. The mosaic of each protein is derived through the superposition of diverse X-ray structures, each of which is obtained with a different organic solvent. The relative position of the red and green shapes is conserved in the two proteins, thus unveiling possible common hotspots.

such methods can often be thought of more as functional groups rather than proper fragments. For this reason, MSCS readouts could also be used in the subsequent stages of the hit-to-lead process.

It has to be noted here that such a technique is not expected to be applicable in all the cases. For starters, the target proteins have to be stable under the crystallization conditions in the presence of organic solvents. Then, the hotspot detection is anticipated to be more effective at solvent-exposed protein sites, owing to the increased turnover between water and organic molecules.

From multitarget fragment to multitarget lead

In a recent review article, Morphy *et al*. [1] have reported that most of the 92 reviewed multitarget ligands were obtained by a 'designing in' approach, whereas only a few of them were generated by a 'designing out' strategy (i.e. from triple-target to dual-target). In light of the highly promiscuous character of fragments one can envision a 'designing out' rather than a 'designing in' approach for fragment-based multitarget drug discovery. However, owing to the low MW of fragments and the possible generation of a mosaic of fragments within protein-binding pockets, the 'designing in' approach, aimed at increasing the MW and therefore compound selectivity, might also be envisioned.

In Fig. 4 a possible strategy for finding multitarget drugs using fragment-based approaches is reported. Depending on the mosaic of hotspots obtained (Fig. 3), fragment- and MSCS-based approaches can be used to generate hybrid, fused or even chimeric (Fig. 1) multitarget compounds. In particular, a possible role for fragment-based methods in multitarget hit identification can be envisioned in the generation of low-molecular-weight hybrid or fused compounds, pointing to this strategy as one of the most promising in polypharmacology. Once the multitarget hit has been designed one should move to the optimization step. This will probably be unfeasible for totally unrelated targets that share only a modest selectivity overlap. Conversely, better outcomes should be expected for closely related targets where similarities at the binding sites offer more room for development. Unfortunately, it can be rather complicated to predict what the optimal *in vitro* activity ratio between targets should be to obtain *in vivo* efficacy [50]. We note that drugs acting on multiple targets should be efficacious even when characterized by low affinity constants toward the targets taken singularly [51]. To this end, the high nM to low μM boundary could be used as a checkpoint. Once reached, the hit progression should move toward PK profiling and tests on animal models, where even a weak inhibition of proteins acting simultaneously on the synergistic pathways of a complex disease could be more effective than selective compounds with higher affinity.

Computer-assisted multitarget drug design

The rise of polypharmacology added new layers of complexity to the already intricate issue of developing molecules active against a single target. In polypharmacology, an effect does not simply spring from a linear combination of independent events involving the same ligand and several targets. Instead, target–ligand associations often affect each other, creating intricate and convoluted patterns, which are difficult to understand let alone predict [52]. For many years now, computational techniques have been part of standard drug discovery processes, used to find new hits or to

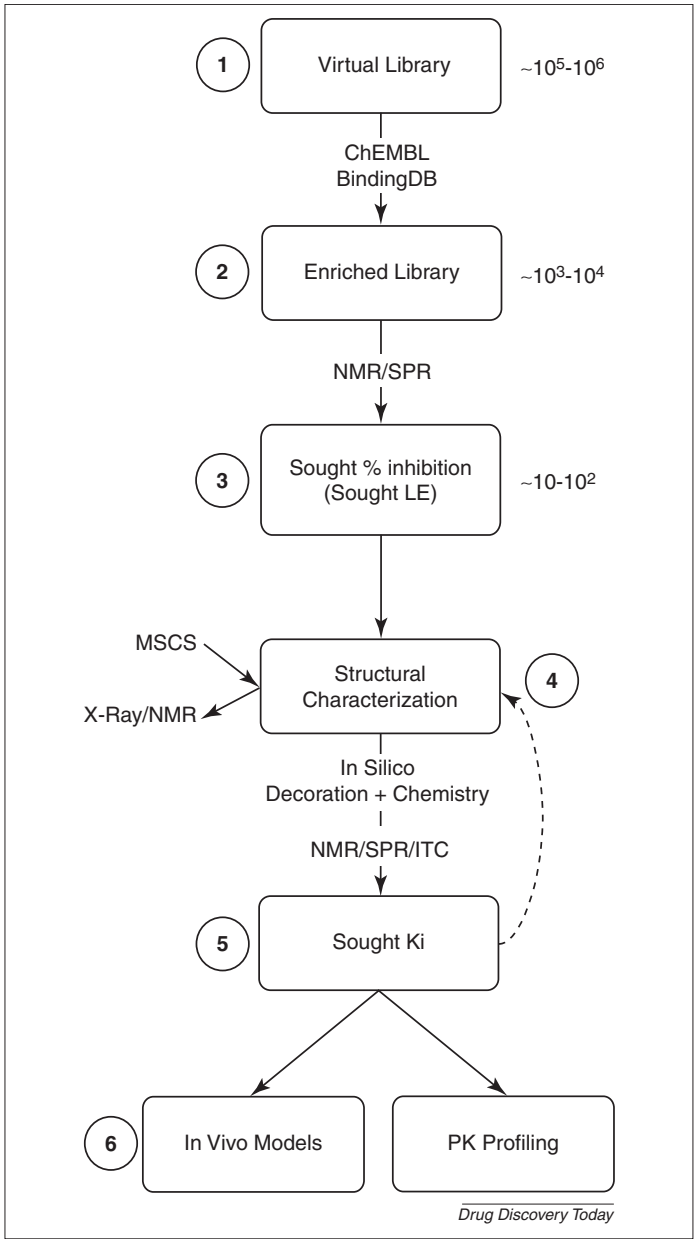

FIGURE 4

Proposed fragment-based pipeline for multitarget discovery. The figure refers to a general example, where the target proteins can be enzymes or ion channels. Starting from a dataset of obtainable fragments ① a library is assembled and enriched with scaffolds found to be already 'accepted' by the targets of interest through the analysis of in-house data and/or publicly available databases such as ChEMBL or BindingDB ②. The primary screening, done via ligand-observed NMR or SPR spectroscopy, leads to a reduced number of compounds showing some multiple inhibitory activity and good ligand efficiency (LE) ③. At this stage, every hit is structurally characterized via protein-observed NMR or X-ray crystallography, whereas multiple solvent crystal structure (MSCS) determinations can feed in structural information to help the fragment progression ④. The selected fragments are grown into bigger molecules by computer-assisted addition of chemically accessible moieties, and assayed via NMR, SPR or ITC. Subsequent phases of optimization are needed to lower the K_i values down to the low μM boundary against all the studied targets ⑤. Preliminary *in vivo* assessments, along with fast PK profiling, should reveal the potential of molecules against a given pathology ⑥.

improve the pharmacological profile of a candidate [53]. It was therefore natural to assume that computational approaches should have been smoothly extended to multitarget drug discovery campaigns. However, although it has been an appealing idea for some time, the application of computer-assisted drug design (CADD) to multitarget drug discovery remains very recent and episodic. Is this because standard protocols are too irrevocably bound to the 'one protein, one disease' idea to keep pace with the ongoing paradigm shift? Or is it just a matter of time before the traditional toolkit of the computational medicinal chemist is efficiently rewired to work with multiple targets? In the remainder of this section we will try to provide some possible answers to these questions. First, we will discuss in detail the role of computational methods in identifying meaningful target combinations. Then, we will report on some multitarget hit discovery strategies. Finally, we will outline some possible future directions.

Defining an optimal target combination

Owing to important technological advances [54–56], metabolic and signaling pathways are being characterized on an unprecedented scale and level of detail. Traditional molecular biology is rapidly giving way to omics biology, a group of specialized disciplines that aim to describe the wholeness of a given biological subject (e.g. genomics is the study of the entire genome of a given organism). Computational methods could be crucial for mining this ever-increasing flow of data, and for identifying target combinations that, if appropriately modulated, could provide a synergistic physiological response (Box 1). In particular, interactomics and pocketomics are emerging disciplines that are gaining increasing importance in polypharmacology. Their development is intimately related to the application of computational schemes.

Interactomics studies networks of protein connections at the molecular level, compiling and comparing interaction maps [57]. From a mathematical perspective, an interaction network can be considered a colored digraph: annotated nodes, which represent the metabolic pathways that connect receptors and enzymes, linked by oriented edges (Box 4). Hence, by regarding these networks in the context of graph theory, it is possible to derive underlying mathematical properties consistent with experimental observations. Simple topographical representations are thus transformed into predictive models [58–60]. Network analysis shows that relevant signaling pathways are usually safeguarded by finely tuned mechanisms of redundancy. This strengthens the idea that the weak but simultaneous modulation of several targets is a conceptually more promising strategy for triggering a physiological response than the potent but narrowly focused inhibition of a single protein [61]. Ideally, this kind of analysis should automatically identify meaningful crossroads and branching points along signaling pathways. This is because these nodes are privileged targets for blocking compensatory metabolic routes. Ultimately, therefore, they hold the key to overcoming a network's strength [8]. The rational development of a multitarget drug turns out to be intimately related to the concept of network biology. However, conclusive results have not yet been provided by initial attempts to select specific target combinations by exploiting interactomics [24]. No clear structural indication toward a specific scaffold or chemical class could be gathered from relationships based solely on molecular biology and biochemistry [62]. Multitarget drugs can exert their activity binding to proteins unrelated from the evolutionary point of view. Moreover, just a small number of mapped nodes have represented actual drug targets [63], making the earliest implementations extremely prone to provide false positives. For this reason, researchers have begun to complement interaction and signaling networks with information gathered from analyzing chemical and pharmacological data. This is in line with the key role that small molecules have in metabolism [64]. This approach was initially limited by the availability of compound libraries experimentally tested on multiple targets [57]. The Similarity Ensemble Approach (SEA), a pharmacological network built by connecting nodes according to the similarity of their binders and independently from their experimentally tested cross reactivity, was a turning point in the field [59]. SEA developers were able to provide an assessment of the random expectation, which, in turn, defines the significance of each connection [65]. In other words, SEA focuses only on meaningful connections that reflect underlying similarities between pharmacological profiles. The model was robust enough to predict the associations of several known drugs with unexpected targets accurately [66]. However, even when a suitable target combination can be identified, the actual development of a multitarget candidate still depends on the possibility of developing a molecule that can physically interact with multiple proteins. This, in turn, depends on the presence in each target of binding regions that can lodge the putative drug.

Pocketomics can be defined as a very specific branch of chemogenomics. Whereas the latter attempts to classify the interactions of all possible chemicals with all possible proteins, pocketomics focuses on those regions of the target where interactions at the molecular level take place [67]. In other words, this discipline studies the shape, size and other physicochemical features of binding sites along with methods and techniques to assess similarities between them. Ligands can bind to dissimilar pockets by adopting alternative conformations or driving interactions involving distinct parts of the same molecule. The presence of intimately related pockets is thus not an indispensable requirement for promiscuous pharmacological activity. Conversely, the opposite is usually true: the presence of very similar pockets probably

BOX 4

Glossary of graph theory.

Graph theory (or theory of networks) is a branch of discrete mathematics concerned with the study of graphs. A graph can be informally defined as a collection of vertices V (also referred to as dots or points) interconnected by edges E (also referred to as lines or links) according to a set of rules. Unless otherwise specified, the term graph usually denotes a simple graph, namely a graph with no loops (i.e. edges always connect two vertices) and no parallel edges; two different edges are never mapped on the same pair of vertices. In a directed graph (or digraph) edges have directions and connect two vertices in a specific order, from the tail vertex to the head vertex. A directed edge is sometimes referred to as an arc or arrow. In a weighted graph a weighting function w is associated with every edge. The term network is usually considered a synonym of weighted graph. Different features (referred to as labels or colors) can be associated with each vertex. Graphs where vertices are annotated in different ways are called colored (or chromatic, or labeled) graphs.

indicates a certain level of cross reactivity, hence the importance of pocket similarity predictive algorithms [43].

Although the first generation of similarity assessment methods relied only on sequence conservation, the current approaches, although differing in practical details and technicalities, all share the same modular approach, which combines three-dimensional and evolutionary traits [68]. First, they provide a simplified representation of the pockets, coding pharmacophoric features and structural determinants in compact data structures. Then, a pair-wise matching procedure computes similarities between different descriptions. These predictive tools are particularly useful when complemented with a functional definition of druggability, namely the propensity of a cavity to accommodate not just every molecule but a molecule with drug-like features [69,70]. In this respect, it should be mentioned that recent computational approaches have been reported by the Barril's group to analyze binding pocket druggability by means of first principle computational approaches [71,72]. In particular, they have mathematically defined an index of druggability that can be applied to novel proteins that do not fall in the main target classes. This approach, as well as the multicopy simultaneous search method (MCSS) proposed by Miranker and Karplus [73], can be particularly suited, at an upstream level of a multitarget drug design workflow, for the identification of common patterns between divergent targets involved in the same disease.

Because of its profound implications for cancer insurgence and development, the kinome (i.e. the complement of human kinases) represents an ideal test case for evaluating the holistic contribution of omics biology to multitarget drug development. Cancer is a multifactorial disease usually generated by the accumulation of genetic insults on multiple genes. With significant exceptions, the pharmacological inhibition of a single kinase does not translate into a lasting antitumor effect. Indeed, some marketed anticancer drugs owe their potency to their ability to interact with multiple kinases, even though they were originally designed for specificity [15]. Members of the protein kinase superfamily share common regulatory patterns and the same three-dimensional fold. However, although obtaining nonspecific (pan) kinase inhibitors is a comparatively easy task, developing kinase inhibitors that only target selected members of the kinome is not so easy. Sequence homology, local conformation at the binding pocket and SARs of ATP-competitive inhibitors vary considerably within the superfamily. Moreover, the three things do not necessarily correlate [74,75]. Metz and colleagues developed a statistically weighted map of the kinome by assembling information from sequence homology and ligand-binding affinity [76]. This network provided an important insight into identifying target combinations: the strength of the connection between two nodes can be maintained, strengthened or almost abolished by resorting to different chemotypes. Therefore, networks complemented by information on structures and binding pocket similarities can not only help predict an optimal target combination but also suggest novel chemical scaffolds likely to provide the sought effect. The recent account by Apsel and colleagues exemplifies the challenges facing those who seek to use advanced tools for predicting polypharmacology target combinations [77]. The authors report the discovery of a series of aryl-substituted pyrazolopyrimidine inhibitors displaying activity against tyrosine kinases, such as Src, VEGFR and Hck, as well as lipid kinases, in particular the phosphatidylinositol-3-OH-kinase (PI(3)K) family. Tyrosine kinases and lipid kinases only share a limited sequence identity and loose structural similarity. In particular, there is a small gatekeeper residue that characterizes tyrosine kinases and that was exploited to achieve specificity for this subfamily [78]. It is not conserved in PI(3)K, where it is replaced by an isoleucine. Only retrospective X-ray crystallography studies could explain how the reported scaffold could display selectivity toward tyrosine kinases and PI(3)K family members. The inhibitor/lipid kinase complex revealed that the activity was caused by three distinctive features: (i) an unprecedented conformational rearrangement of the isoleucine gatekeeper; (ii) the ability of the inhibitor to adopt different bound poses at the hinge region; (iii) and the presence of a specific interaction with a conserved glutamate. Because pocket similarity is elusive here, and because Src and PI(3)K can be easily connected biochemically because they belong to the same signaling pathway, the combined application of network biology and structural chemogenomics emerges as a very promising strategy for detecting target combinations.

Multitarget virtual ligand screening as a hit identification strategy

Once a suitable combination of targets has been identified and validated, a rational drug design project can begin identifying multitarget hits. In a single-target endeavor, HTS would represent a straightforward strategy for identifying initial hits. Although powerful, HTS is costly in terms of resources, time and personnel. This is true when just one target is involved. The costs increase sharply if multiple targets are to be considered simultaneously.

Virtual ligand screening (VLS) represents a fast and efficient alternative to HTS for processing large libraries of compounds [79]. In single-target VLS (Fig. 5a) every molecule in the library is tested against an ideal model of activity. This model can be based on pharmacophoric and physicochemical descriptors extracted from known ligands or on interactions at the target binding site. Ligand- and structure-based models can be used independently or in combination. Each compound is assigned a predicted activity score and the library is ranked accordingly. Only the top-ranking fraction proceeds to further testing. The most straightforward way to apply VLS in the multitarget framework is to apply the screening protocol to each target independently [13]. Although VLS applied to multitargets can provide useful information for identifying hybrid, fused or chimeric hit compounds, the most straightforward results coming from a multitarget VLS campaign could be the identification of hybrid molecules able to bind simultaneously to the selected targets. In fact, in the next step of a possible VLS-based workflow, the researchers must somehow combine and analyze the generated results to decide which molecules to prioritize for testing, based on the previously mentioned cornerstone of polypharmacology: a weaker activity, as long as it involves multiple targets, is preferable to an activity that is potent but limited to a single protein [51]. An experimental multitarget profile is likely to emerge from those molecules that, even if they do not reach the top-ranking fraction in any single run, score on average adequately well and never drop below a given threshold (Fig. 5b). The work of Wei and coworkers represents a good case study in the practical application of multitarget VLS [80]. The authors successfully

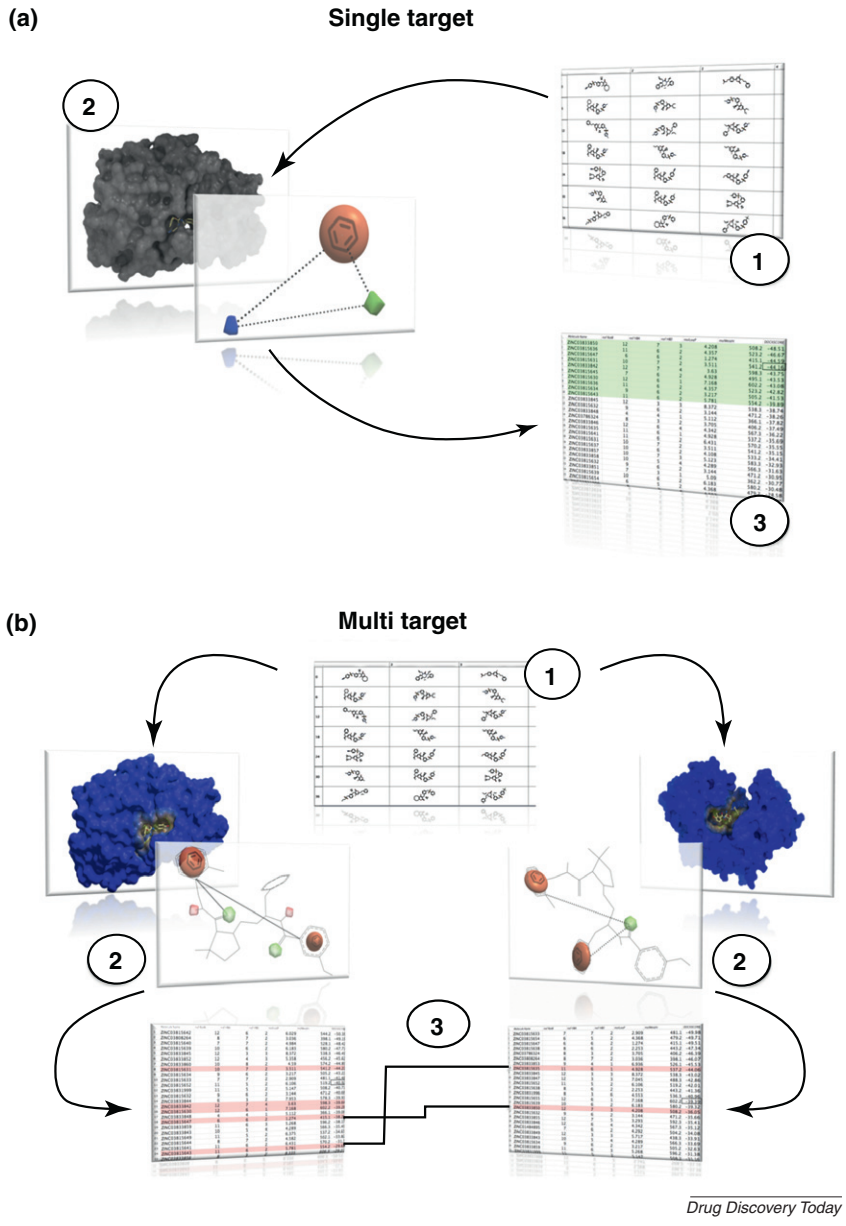

FIGURE 5

Single-target and multitarget virtual ligand screening approaches. **(a)** In single-target virtual ligand screening (VLS) a compound library ① is systematically screened via ligand- or structure-based methods, or both ②. Each compound is assigned a predicted activity score and the library is ranked accordingly. Molecules in the top-ranking fraction ③ proceed to further testing. **(b)** In multitarget VLS the same library of compounds ① is screened independently against different targets ② and the overlapping hits ③ proceed to further testing.

identified novel anti-inflammatory candidates displaying activity against phospholipase A2 (PLA2) and human leukotriene A_4 hydrolase (LTAH4-h). First, they devised a common pharmacophore that combined relevant features from both targets. Then, they carried out independent structure-based VLS runs, filtering out all conformations that did not match the common pharmacophore. Notably, none of the compounds eventually reported to be active would have been identified by simply testing top-ranking molecules.

Kernel methods and, in particular, support vector machines (SVMs) are versatile and efficient strategies already used in single-target screening processes. They are emerging as particularly suitable tools for multitarget-oriented campaigns [81]. The main downside of combined approaches is that, because every screening paradigm is prone to errors, the combination of multiple screening runs is bound to increase the number of false positives significantly. Ma and colleagues demonstrated that, at least for structurally related targets such as kinases, it is possible to train multiple SVMs using only single-target inhibitors and to identify dual inhibitors by combining common hits [82]. They still used multiple runs, but they managed to discriminate between unspecific pan inhibitors and inhibitors specific for a given target combination. Moreover, if the training is performed on known multitarget compounds, it is possible to bypass the need to process the same library multiple times. However, this

straightforward approach strongly limits the applicability of SVMs when studying unprecedented target combinations [83]. Ideally, it should be possible to devise a supervised learning approach to predict directly multitarget compounds that can be trained using sets of specific inhibitors only.

Possible roles for in silico *strategies in multitarget hit-to-lead and lead optimization*

The application of *in silico* techniques after hit discovery will be another challenge for future CADD studies. So far, not much has been reported. Accounts of these kinds of studies are limited to inhibitors acting on closely related targets. Ligand docking and molecular dynamics were successfully applied in SAR studies on dual c-Src/Abl and dual EGFR/VEGFR2 kinase inhibitors [84–86]. Docking also helped rationalize the inhibitory profile of triple angiokinase inhibitors [66]. The concept of balance will probably have a pivotal role in attempts to improve potency toward multiple targets without compromising the LE and the PK profile of the candidate [50]. In this regard, we can envisage a new generation of computational tools that will automatically address the optimization of a multitarget drug in terms of scaffold morphing, introducing modifications that are beneficial not just for one target and tolerated by the others but that enhance the compound profile with respect to all targets at once.

Concluding remarks

The multitarget is a novel and emerging drug discovery paradigm based on the idea that superior therapeutic efficacy and safety can be achieved by designing individual new chemical entities that can simultaneously target different points of a given pathogenic cascade. The enhanced efficacy of multitarget drugs could also be as a result of their preventing unwanted compensatory mechanisms, which might result in cellular redundancy, from developing. In fact, redundant mechanisms can activate alternative pathways, thus impairing the drug efficacy achieved by modulating a single-protein activity. This is the major reason for using drug combinations in several different therapeutic areas. In this respect, multitarget drugs provide a valuable alternative to cumbersome and risky drug cocktails [87].

A multitarget approach to discovering innovative medicines is necessitated by the multifaceted nature of several complex diseases, including neurological and neurodegenerative disorders, cancer and infectious diseases. For these diseases, single-target agents seem unlikely to be the source of future drugs. In fact, the multidimensional view of diseases is replacing the linear causality model based on the 'one disease, one gene, one target' and the 'one single-target drug' paradigms. A first step toward this change is the multitarget or, even better and more affordable, the dual-target strategy, where two targets at different key points within the same or concurrent pathogenic pathways are carefully chosen for their potential additive effects or synergistic potentiation. However, the rational design of multitarget drugs faces considerable challenges. These arise from the need for new methods to validate target combinations and to identify preliminary hit compounds. Here, we have shown that fragment-based and computational approaches can play a major part in the target selection, hit identification and hit-to-lead steps of the drug discovery process. As a consequence, they could significantly help overcome attrition in the very early stages of this process. This would reduce the 'resource-hungry' nature of multitarget efforts, which is the key factor in limiting their wider application to drug discovery.

Another issue to be re-examined within the multitarget framework is the classic meaning of SARs. Optimizing multitarget SARs, while maintaining drug-like properties, could be challenging. The simplest possible scenario is that improving the biological profile of a molecule toward a first target could decrease its activity toward a second. In this respect, new generation statistical analyses could be valuable in defining quantitative SARs and driving the design of new multitarget drugs. Then, in lead optimization, it will be another challenge to equalize multiple activities while keeping drug-like properties and controlling unwanted off-target effects. However, the ultimate goal would be treating the complexity of the biological system, and its response to the action of drug. This new research field, dubbed network pharmacology [8], offers the promise of tackling the two major sources of attrition in drug development: efficacy and toxicity.

We conclude by noting that, given the crucial need in the pharmaceutical industry for new disease-modifying chemical entities in several therapeutic fields, the multitarget strategy offers a new framework for thinking about how to innovate in drug discovery – thus, its time has come.

Conflicts of interest

The authors declare no conflict of interests.

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