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Paper

Weak-binding molecules are not drugs?—toward a systematic strategy for finding effective weak-binding drugs

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Abstract

Designing maximally selective ligands that act on individual drug targets with high binding affinity has been the central dogma of drug discovery and development for the past two decades. However, many low-affinity drugs that aim for several targets at the same time are found more effective than the high-affinity binders when faced with complex disease conditions, such as cancers, Alzheimer's disease and cardiovascular diseases. The aim of this study was to appreciate the importance and reveal the features of weak-binding drugs and propose an integrated strategy for discovering them. Weak-binding drugs can be characterized by their high dissociation rates and transient interactions with their targets. In addition, network topologies and dynamics parameters involved in the targets of weak-binding drugs also influence the effects of the drugs. Here, we first performed a dynamics analysis for 33 elementary subgraphs to determine the desirable topology and dynamics parameters among targets. Then, by applying the elementary subgraphs to the mitogen-activated protein kinase (MAPK) pathway, several optimal target combinations were obtained. Combining drug–target interaction prediction with molecular dynamics simulation, we got two potential weak-binding drug candidates, luteolin and tanshinone IIA, acting on these targets. Further, the binding affinity of these two compounds to their targets and the anti-inflammatory effects of

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them were validated through *in vitro* experiments. In conclusion, weak-binding drugs have real opportunities for maximum efficiency and may show reduced adverse reactions, which can offer a bright and promising future for new drug discovery.

Key words: weak-binding drug; polypharmacology; mathematical modeling; systems pharmacology

Introduction

Over the past decades, the dominant paradigm in drug discovery has been to develop maximally selective ligands that act on individual drug targets with high binding affinity [1]. However, analysis of the binding efficiencies of natural products and marketed drugs indicates that therapeutic efficacy is not necessarily associated with high binding affinity [2]. For instance, memantine, a drug for Alzheimer's disease, is a small, low-affinity, nonselective N-methyl-D-aspartic acid (NMDA) receptor antagonist, which shows a lower prevalence and less side effects than high-affinity, single-target drugs [3]. The underlying regulatory mechanisms of many effective low-affinity drugs have yet to be fully characterized [4]. Particularly, natural products, many of which having weak binding affinity, have been proved to have solid therapeutic efficacy from earlier animal-based drug discovery settings [5, 6]. It is estimated that there are currently ~110 000 low binding affinity small molecules (with inhibitory constants to their targets more than 10^{-6} M) in the public database [7]. However, the potential efficacy of these weak-binding molecules have largely been ignored.

There are a number of reasons of why weak-binding molecules have been neglected for a long time as a source for drug discovery in biological sciences. First, there is still a paradigm in the minds of many scientists stating that effect and specificity only come from drugs that bind tightly to a target molecule [8]. It has been envisioned that a weak binder is not specific to its target, and typically shows high cross reactivity to other binding sites. However, cross reactivity of the drug may not be a disadvantage, and it gives the drug a chance to interact with multiple targets for maximum efficiency [8]. Second, for weak-affinity molecules, the amount of binding to the target can be perceived as a problem, as it may be too low to propel a response. However, if local concentrations of a weak binder are high enough, it can drive the equilibrium, resulting in considerable bound ligand [8]. Third, most of the current drug discovery models are incapable of screening or analyzing weakly binding drugs or weak biological interactions. As the molecular libraries are typically screened at micromolar concentrations or lower, the weak-binding molecules can be easily washed out in a variety of screening processes.

To re-recognize the importance of weak-binding molecules as well as understand the mechanisms of action of them, we should define and predict the drug phenotype response on the basis of the quantitative and systematic analysis of drug–protein interactions on a proteome-wide scale. The immediate question to address is how we can select the correct combination of therapeutic targets within complex molecular networks and rationally design weak-binding drugs.

Recently, network pharmacology approaches are emerging as a powerful way to re-purpose approved drugs and elucidate the mechanisms of action of natural products. Tang *et al.* [9] depicted a number of network-based computational-experimental approaches for searching potential drug target combinations in the disease-associated networks. They also gave representative examples of how system-level network approaches may lead to multi-target therapies that are less vulnerable to drug resistance and side effects in anticancer drug discovery. Kibble *et al.* [10] presented a similar network pharmacology approach to map the unexplored target space and therapeutic potential of natural products. Using drug and target interactions deposited in DrugBank database, Barneh *et al.* [11] constructed and analyzed the drug–target network and comprehensively assessed the evolutionary changes in the networks following expansion of DrugBank database from version 1.0 to 4.0 [12, 13]. Compared with the pioneering study conducted by Yildirim *et al.* [14], they showed that such advances in database quality can better meet the needs of modern pharmaceutical industry. The above studies [9–11] provided good examples of the application of network pharmacology in drug discovery. However, most of them only performed static analysis for the networks, and few have considered the dynamics properties of drug–target interaction and target–target interaction, which should be key points in understanding the efficacy and action mechanisms of weak-binding drugs.

In this review, we intend to draw attention to the discovery of this new range of drug candidates characterized by weaker binding and/or faster kinetic profiles. Toward a systematic understanding of effective weak-affinity molecules, it is important to emphasize that weak binders typically demonstrate a dynamic binding profile possibly with high dissociation rates. Estimates of the duration of drug–receptor residence time can be indicative of drug performance. In addition, a weak-binding molecule is more likely to bind to a number of different targets, and the network topology and dynamics properties among these targets can be a key contributor to the efficacy of the weak-binding drugs. Further, we also discussed the current available tools to screen for weak-binding drug candidates and propose a systematic strategy for analyzing and discovering effective weak-binding drugs.

Weak-binding drugs: affinity and kinetics

Features of interacting drug–target pairs provide useful information on the strength and kinetics of binding. Binding kinetics is concerned with the rate constant of ligand association (k_{on}) and ligand dissociation (k_{off}) [15]. At equilibrium, the ratio of the dissociation to the association rate constants establishes the equilibrium dissociation metric of the ligand ($K_d = k_{off}/k_{on}$), which is a usual measure of affinity and determines the fraction of receptor occupancy at specific ligand concentrations. K_d, k_{off} and k_{on} are intrinsic to the target–drug interaction in question. For a weak non-covalent interaction, K_d is more than approximately 10^{-6} M (usually in the range of 10^{-5} to 10^{-3} M). For many weak or transient interactions, k_{off} is higher than 0.1 s^{-1} [8]. This means that dissociation of the complex is more rapid than seconds. For instance, the drug memantine shows binding to the NMDA receptor in the millimolar range with an off-rate of approximately 0.4 s^{-1} [16]. Fast off-rates of weakly binding drugs could be a key factor in designing effective ion-channel blockers, and that this principle can apply to a number of neurological and other targets [8]. Actually, the equilibrium

dissociation constant K_d, measured *in vitro*, is not always directly related to the *in vivo* efficacy of a ligand. The residence time of a drug molecule on its molecular target has been proposed to be more crucial for sustained drug efficacy *in vivo* than the affinity of the drug for its target [17]. The residence time is related only to the rate of complex dissociation, which is the reciprocal of the dissociation rate constant ($1/k_{off}$). Unlike k_{on}, which is limited by the diffusion rate in physiological solutions and affected by *in vivo* pharmacological factors, k_{off} is entirely dependent on specific interactions (such as changes in protein conformation, nonpolar forces, hydrogen bonds, van der Waals interactions and so on) between the ligand and its target binding pocket [18]. Therefore, optimization of such interactions, and consequently increasing the dissociation rate of a ligand, may be the fundamental value of medicinal chemistry in terms of designing weak-binding drugs.

Weak-binding drugs: polypharmacology

Finding drug candidates that selectively (at high affinity) bind single targets has been successful for diseases with a clearly defined mechanism, etiology and pathophysiology [19]. However, when faced with complex disease conditions, such as cancer, depression and cardiovascular diseases, 'promiscuous' or 'dirty' drugs aiming for several targets at the same time could be far more productive than those single-target drugs [20, 21]. Complex diseases are not caused by single molecular defect, but are rather the result of a combination of molecular dysfunctions [22]. In this context, multi-target drugs may have a better chance of affecting the complex equilibrium of whole cellular networks [23]. In fact, polypharmacology, which focuses on designing drug to multiple target receptors, has emerged as a new paradigm in drug discovery [24]. Polypharmacology currently encompasses both multiple drugs that act independently on different targets, and a single drug binding to multiple targets within a biological network, as opposed to the concept of 'one gene, one drug, and one disease' [25–27]. In recent years, the efficacy of multi-target drug is supported by observations concerning the robustness and resilience of complex biological systems. For example, most approved kinase drugs potently inhibit multiple targets, and they are attractive therapeutic agents for numerous disorders ranging from neurology to cancer [28]. Development of a multi-target drug is likely to produce a drug binding to its targets with weak affinity because it is unlikely that a small, drug-like molecule will bind to a variety of different targets with equally high affinity [23]. In other words, cross reactivity of the drug should be substantial so that it can theoretically interact with multiple targets for maximum efficiency [8]. Partially affecting several targets by a low-affinity, multi-target drug rather than completely eliminating the links can also increase weak links in cellular networks and stabilize these networks [4, 29]. Moreover, through including weak multi-target drugs, the size of drug-amenable targets will increase significantly in terms of potential druggable proteins. Databases on cellular and protein networks will therefore show potential to define new targets for drug design [23].

Tools for screening weak-binding drugs

Experimental tools

To find a single target, usually a protein playing a major role in the disease process, and then to find the high-affinity binders for this target has been the central dogma of drug discovery and development. Current tools available to the pharmaceutical industry to identify new drugs rely heavily on high-throughput screening (HTS) procedures, where large libraries, consisting of hundreds or thousands of molecules, are tested for their binding to specific targets [30]. Even though HTS has been a success in some cases, it is now abundantly clear that it has been a disappointment in many drug discovery projects. The causes of attrition in later phases of drug discovery have been attributed to poor absorption, distribution, metabolism, excretion and toxicity (ADME/T) properties [31]. Screening for weak-binding drugs, while promising, is still a challenge considering current drug screening approaches. Generally, weakly binding drugs/weak biological interactions are not studied because of difficulties in screening or analyzing them. Most HTS assays rely on indirect detection methods, such as fluorescence, absorbance or radioactivity that could be a barrier for estimating weak-binding events. Because of limitations in assay design, HTS procedures can produce false positives and negatives, especially when estimating the presence of weak binders. Nevertheless, HTS based on inhibition assays of enzyme activities, for example, can, if properly designed, detect weak binding of compounds with half maximal inhibitory concentration (IC_{50}) less than 10^{-4} M [32]. There are a number of potential methods for screening weak binders to protein targets, such as nuclear magnetic resonance [33], mass spectrometry [34], X-ray crystallography [35], affinity chromatography [36], capillary electrophoresis [37] and surface plasmon resonance [38]. As to G protein-coupled receptor (GPCR) drug discovery, the vast majority efforts have relied on cell-based assays coupled with HTS of large compound libraries for hit identification [39].

Computational tools

The process of defining and predicting polypharmacological effects of weak-binding drugs requires a quantitative understanding of the structure and function of a protein, as well as an understanding of the protein's interaction with small molecules in the context of biological networks [24]. Cellular components carry out their biological functions through interacting with each other in a network-like manner [40]. The network structure along with the dynamics properties largely determine the biological function of the interacting molecules, so that the structure of the biological network involving the drug targets may help to reveal the action modes of weak-binding drugs [41, 42]. Mathematical models of many disease-relevant pathways have been developed with the potential to elucidate underlying disease mechanisms and to identify effective treatment strategies [43, 44]. Properties of these disease-related molecular networks can be analyzed to find potential drug targets and to understand the interaction pattern between them [45, 46]. For instance, the special signaling elements, such as the PI3 kinase [47], the Akt kinase [48] and the insulin receptor substrate family [49], which are important junctions of multiple signaling pathways, have been used as important targets for drug development. The modeling of network behavior has also indicated that the partial inhibition of several targets can be more efficient than complete inhibition of a single target [4]. Further, to quantitatively analyze protein–ligand interactions, one can start with characterization of the thermodynamics and kinetics of protein–drug interactions [15], followed by determination of the conformational and chemical states of proteins on drug binding through allosteric or orthosteric interactions [24, 50]. This process is

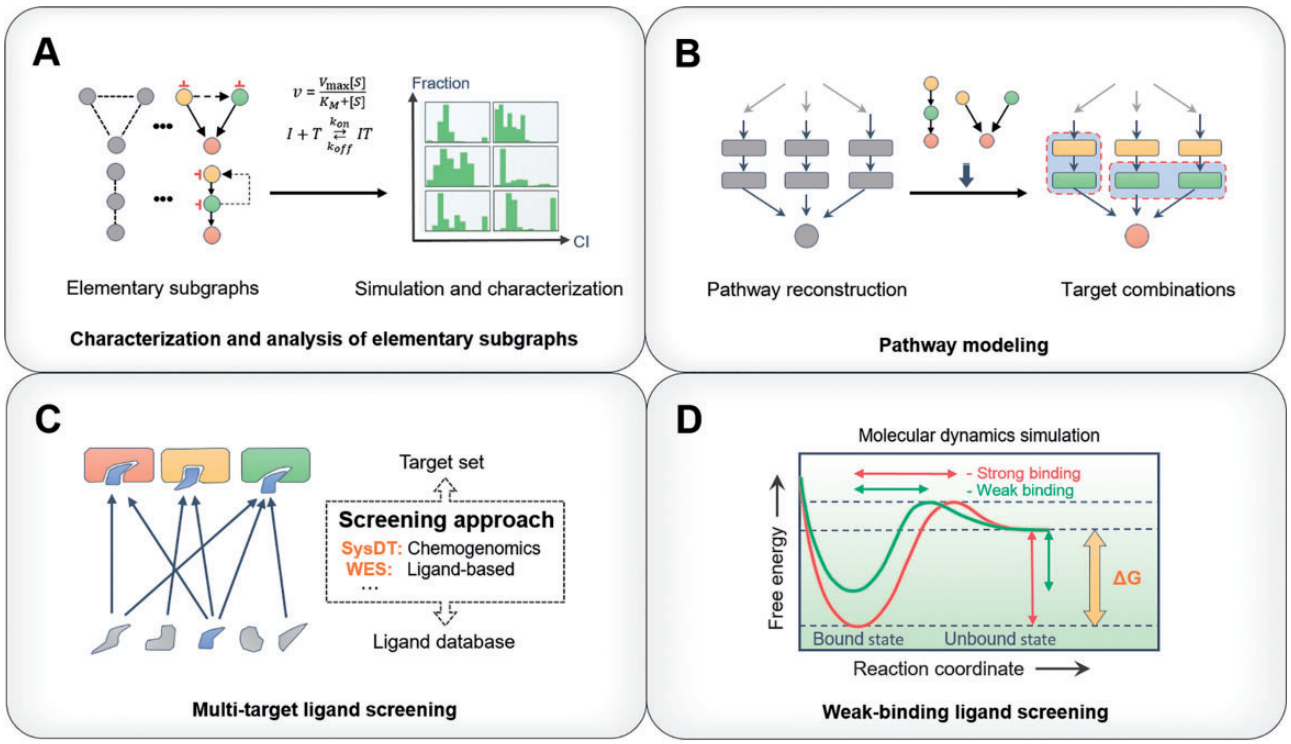


Figure 1. A systematic strategy for weak-binding drug discovery. (**A**) Elementary dynamics analysis reveals the influence of network topology and dynamics parameters on the effects of drugs. (**B**) Applying the elementary subgraph to a specific pathway to find the optimal target combinations. (**C**) Drug–target interaction identification for screening ideal multi-target compounds. (**D**) Assessing drug–target binding affinities via molecular dynamics simulation.

based predominantly on protein–ligand docking [51], free energy calculations [52] and molecular dynamics simulations [53] for the protein–ligand complex.

A systematic strategy for weak-binding drug discovery

To explore new frontiers in pharmacology and rationally design weak-binding drugs, it is necessary to integrate those disjointed computational and experimental techniques into a unified framework. Combining pathway and network analyses, proteome-wide prediction of drug–target interactions and pharmacokinetic and pharmacodynamic models will enable the development of a systematic approach for weak-binding drug discovery. Here, we present a systems dynamics method for inferring network models and predicting the response of cell signaling networks to multi-node weak perturbations (Figure 1). Specifically, we first performed a dynamics modeling for 33 elementary subgraphs to investigate the influence of network structure and dynamics parameters on the effects of multi-target drugs. Second, the elementary subgraphs were applied to a classic mitogen-activated protein kinase (MAPK) pathway to search for the optimal target combinations. Then, based on these target combinations, two in-house drug targeting approaches, SysDT [54] and WES [55], were used to screen the multi-target compounds from both small molecule drugs and natural products. To evaluate the binding affinity between the targets and compounds, we performed the molecular dynamics simulation and calculated the binding free energy between them. These predictions were then tested by kinase inhibition assays. Finally, we validated the potential therapeutic effects of these weak-binding drug candidates by *in vitro* experiments.

A case study: discovering effective weak-binding drugs acting on MAPK pathway

Elementary dynamics analysis reveals the topological characteristics and dynamic properties of target subgraphs

To search for the optimal drug target combinations for weak-binding drugs, as well as the network topologies and dynamics parameters these targets involved in, we first built dynamics models for a series of three-component elementary subgraphs, which could be considered as simplifications of molecular networks [41, 56, 57]. The elementary subgraphs were first extracted from two previous studies [58, 59], and then extended to contain all possible interactions between the components (Supplementary Figure S1). Each of the elementary subgraphs represents one type of basic signal transmission pattern of a target combination. The two drug targets *A* and *B* in an elementary subgraph can propagate the signal to a downstream effector *C* whose activity is a measure of the therapeutic effect. Relationships between the targets *A* and *B* can be activation or inhibition and may contain feedback loops, while *A* and/or *B* has either promotion or suppression effect on the efferent component *C*. There are in total 33 elementary subgraphs, which are divided into two groups: the 'single-tandem subgraphs' (STSs) and 'dual-parallel subgraphs' (DPSs). In the STSs, *A* and *B* are in one single pathway, where the effector *C* is directly affected by target *B*, and *A* has indirectly influence to *C* through *B*. While in the DPSs, the two targets are in two parallel pathways and can both directly influence the effector *C* (Supplementary Figure S1). Among these subgraphs, some are commonly found in intracellular signaling networks, such as the cascades [60], feedforward loops [61, 62] and feedback loops [63], and the specific biological

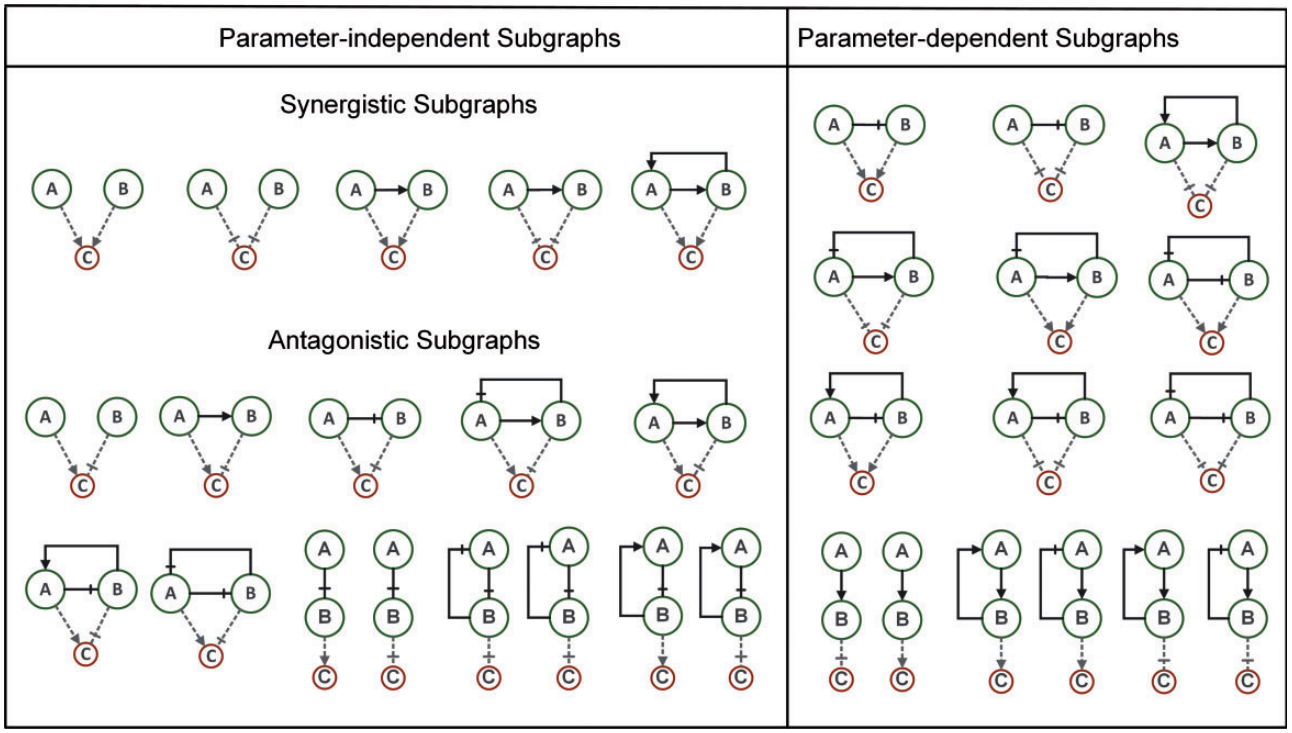


Figure 2. The 33 elementary subgraphs and dynamics modeling analysis of their synergistic effects. The elementary subgraphs can be parameter-independent subgraphs, which means the synergistic/antagonistic effect of the subgraph is only dependent on the network structure, but not dependent on the dynamics parameters (K_M, k_{cat}) in the subgraph. These parameters (K_M, k_{cat}) depict the underlying kinetic rates and dynamic properties of the signaling pathways. The other group of subgraphs are parameter-dependent subgraphs, the synergistic/antagonistic effect of which depend heavily on the dynamics parameters.

functions they carry out have been well discussed in previous studies [60–63]. Although many biological signaling networks may conform to one of these simple topologies, others may be abstracted to one that recapitulates the physiologically relevant emergent properties [59].

The elementary subgraph was then modeled by a set of ordinary differential equations (ODEs) derived by the rate laws of mass action and the complete Michaelis-Menten reaction kinetics (Supplementary Figure S2). For each elementary subgraph, the value of Michaelis constant (K_M) and catalytic constant (k_{cat}) were generated by Latin hypercube sampling in a biological range of 0.001, 10 mM for K_M, and 0.1, 10 s for k_{cat} [56]. A total of 10 000 parameter sets were generated randomly to explore the activity of C under simultaneously inhibition to the two targets for different parameter sets.

To evaluate whether the two targets A and B in an elementary subgraph can have synergistic effect, we supposed that there are two individual inhibitors I_A and I_B, respectively, binding to targets A and B, and calculated the combination index (CI) to distinguish the elementary subgraph between synergy and antagonism [56, 58].

$$CI = \frac{[I_A]_{combination}}{[I_A]} + \frac{[I_B]_{combination}}{[I_B]}$$

Where $[I_A]$ and $[I_B]$ are the concentration of I_A and I_B that individually achieve 50% inhibition effect on C, and $[I_A]_{combination}$ and $[I_B]_{combination}$ are the concentration of each inhibitors producing the same 50% effect when used in combination. For each set of parameters, the minimum CI value for synergistic cases (or maximum for antagonistic cases) was extracted and used for drawing the CI distributions for the elementary subgraphs (Supplementary Figure S3). When $CI < 1$ or $CI > 1$, the elementary subgraph is considered as a synergistic or antagonistic subgraph, respectively.

The distribution of CI values of the 33 elementary subgraphs under varying parameters shows that the synergistic effect of 18 subgraphs is only determined by the network topology, rather than the dynamics parameters (Figure 2 and Supplementary Figure S3). There are five parameter-independent synergistic subgraphs, and they are DPSs in which targets A and B have the same promotion/suppression effect on effector C. The other 15 subgraphs are parameter-dependent subgraphs, in which the dynamics parameters (K_M, k_{cat}) conferring a major influence on the synergistic effect of the subgraphs (Figure 2). Overall, the synergistic/antagonistic effect of elementary subgraphs can be determined either by the network topology alone, or by both of the network topology and the dynamics parameters.

Applying elementary subgraphs to the MAPK pathway to find optimal target combinations

Among numerous intracellular signaling, the MAPK cascades are evolutionarily conserved and well-studied signaling pathways that play a key role in the regulation of fundamental cellular processes in responses to stress and inflammation [64, 65]. Applying the elementary subgraphs for experimentally testable system, we reconstructed a condensed MAPK signaling network, in which any intermediate or auxiliary process poorly defined was excluded (Figure 3). Using a set of parameters within appropriate biological ranges, the modeling work was carried out by numerical integration of ODEs (Supplementary Table S1). The reconstructed system can be activated by a LPS-

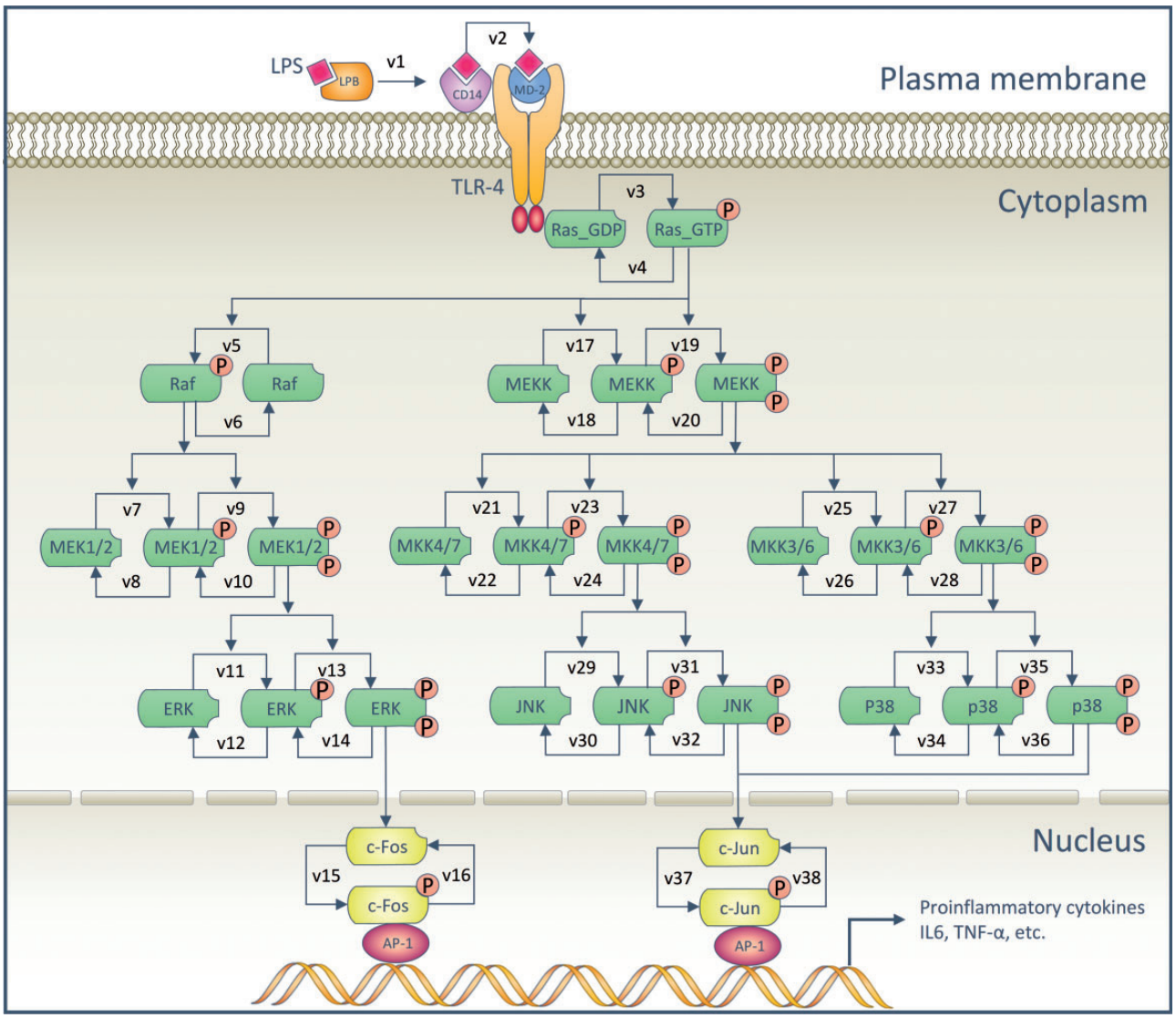


Figure 3. Mathematic modeling of MAPK pathway. Detailed equations and parameters can be found in the Supplementary Table S1.

induced stimulation. The perturbation effects (inhibitory effect of drug) on different subgraphs were then evaluated by assessing the signal levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α.

There are 15 target combinations (corresponding to 3 STSs and 12 DPSs) among the six targets (i.e. MEK1/2, MKK4/7, MKK3/6, JNK, ERK and p38) in the MAPK pathway (Table 1). To assess the efficacy of weak-binding drugs on these elementary subgraphs, we simulated the inhibitory effects of two types of perturbations: multi-weak perturbation (inhibit two targets simultaneously with 20% inhibition rate) and single-strong perturbation (inhibit a single target with 80% inhibition rate).

The results show that among multi-weak perturbations on the 3 STSs, only the combinatory inhibition of MKK3/6 and p38 shows desirable inhibitory effects on IL-6 and TNF-α, which is almost equivalent to the single-strong perturbation on each of the targets alone (Table 1). Compared with the other two branches, the multi-weak perturbation on JNK pathway branch (MKK4/7 + JNK) shows little influence on IL-6 and TNF-α production, consistent with previous research showing that the JNK signaling pathway is not essential for TNF-α gene expression in embryonic fibroblasts [64, 66]. For the 12 DPSs, simultaneously inhibiting ERK and p38 shows the best inhibitory effects on IL-6 and TNF-α. On the contrary, multi-weak perturbation on MEK1/2 and MKK4/7 shows the least attenuation of IL-6 and TNF-α (Table 1). An interesting observation is that the five optimal target combinations for multi-weak perturbations all contain the p38 MAPK. p38 MAPK is a primary target of anti-inflammatory pyridinyl imidazole drugs that inhibit endotoxin-stimulated production of TNF-α [65, 67]. Moreover, a recent study shows that oscillation of p38 activity is necessary for efficient expression of pro-inflammatory genes such as IL-1β, IL-6 and TNF-α [68].

Collectively, for the MAPK pathway, target combinations containing the p38 kinases are potential priorities for designing weak-binding drugs.

Combining drug–target interaction identification and molecular dynamics simulation for screening effective weak-binding drugs

A compound database, which includes 12 144 natural products from TCMSP [69] and 7391 small molecular drugs from

Table 1. The simulated inhibitory effects of the two types of perturbations on elementary subgraphs or single targets in the MAPK pathway

Perturbations (inhibition rate)	Target combinations	Inhibitory effects (%)	
		IL-6	TNF-α
Multi-weak perturbation: STS (20% for each target)	MEK1/2 + ERK	19.88	14.84
	MKK3/6 + p38	**85.60**	**73.85**
	MKK4/7 + JNK	6.38	4.67
Multi-weak perturbation: DPS (20% for each target)	MEK1/2 + MKK4/7	0.52	0.38
	MEK1/2 + MKK3/6	11.09	8.14
	MKK3/6 + MKK4/7	10.70	7.85
	MKK3/6 + ERK	35.96	26.91
	MKK4/7 + ERK	17.53	13.07
	MEK1/2 + JNK	6.75	4.95
	MKK3/6 + JNK	20.64	15.17
	MEK1/2 + p38	**86.20**	**74.93**
	MKK4/7 + p38	**85.29**	**73.34**
	JNK + ERK	28.22	21.05
	JNK + p38	**85.56**	**73.80**
	ERK + p38	**97.35**	**96.91**
Single-strong perturbation (80%)	MEK1/2	73.83	60.08
	MKK3/6	**87.29**	**76.90**
	MKK4/7	3.34	2.44
	JNK	43.55	32.38
	ERK	75.64	62.13
	p38	**87.56**	**77.42**

Target combinations with IL-6 inhibitory effects >85% are marked as bold.

DrugBank [13], was established for inverse screening. The targets of these compounds were predicted using the SysDT [54] and WES [55] models as we previously described. As we need to screen inhibitors for the MAPK pathway, the action modes (activation or inhibition) between the compounds and the targets are then predicted using a PreAM model [70]. Then, compounds that inhibit more than one protein kinase target of MAPK pathway were filtered in accordance with the three target combinations (ERK + p38, JNK + p38 and MEK1/2 + p38), and 32 molecules were obtained as multi-target compounds (Supplementary Table S2). To evaluate the affinity of these compounds, molecular dynamics simulation and Molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) methods were used to calculate the binding free energies. Briefly, the crystallographic co-ordinates of the four MAPKs were retrieved from the Protein Data Bank (www.rcsb.org) [71], and the compounds were docked to the original ligand binding pocket of each protein. The docked complex with optimal conformation served as starting structure for molecular dynamics simulation according to our previous work [72]. After 5 nanosecond (ns) molecular dynamics simulation, the last 1ns trajectory file was extracted to binding free energy calculation using MM-PBSA module.

The results show that most of the natural products tend to weakly bind to the target proteins with large binding free energy ($\Delta G_{bind} > -25$ kcal/mol) (Supplementary Table S2). Luteolin and tanshinone IIA were selected from natural products database for affinity determination (scopoletin as negative control) and four selective inhibitors for MEK, ERK, JNK and p38, respectively, were selected for comparison (Table 2). The binding affinities of luteolin to the four MAPKs was tested experimentally by *in vitro* kinase inhibition assays (Supplementary Figure S4). It turns out that luteolin possesses weak affinity ($IC_{50} > 10$ μm) to all the four MAPKs. Further, the pharmacokinetic properties of luteolin and tanshinone IIA obtained from the TCMSP database show that both of them comply well with Lipinski's rule of five, which suggests that they can manifest proper biological effects in *in vivo* systems (Supplementary Table S3).

To develop experimentally testable predictions, we simulated the inhibitory effects of the two natural products (luteolin and tanshinone IIA) and the four known selective inhibitors on IL-6 and TNF-α production using the same MAPK pathway model mentioned above (Supplementary Table S1). Computationally, we simulated the effect of each compound at five doses and used the levels of IL-6 and TNF-α as feature metric for MAPK-mediated inflammatory responses to select treatment conditions from the resulting data set that had desirable anti-inflammatory effects. The inhibition rates of the compounds for each target protein were inferred from their IC_{50} curves. The simulation results show that luteolin achieves better effect than all the four selective inhibitors when concentrations are around 10 μM (Figure 4A).

To further experimentally test these predictions, we examined the levels of IL-6 and TNF-α in the supernatant of THP-1 cells under the treatment of these compounds. Briefly, the THP-1 cells were stimulated for 48 h with 25 ng/ml PMA and differentiated THP-1 cells were treated 4 h with LPS (1 μg/ml, as a positive control) or LPS in the presence of the compounds. The IL-6 and TNF-α concentration of supernatant were determined using ELISA kit (see Supplementary methods for more details). The result shows that, pretreatment with luteolin at final concentrations ranging from 1 to 10 μM exhibited a steeper dose-dependent rise in inhibition of IL-6 and TNF-α in contrast to the shallow rise seen with selective inhibitors of MAPK (Supplementary Figure S5).

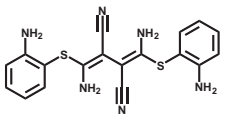
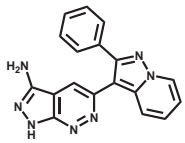
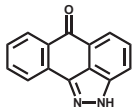
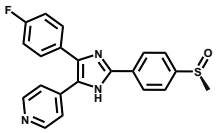
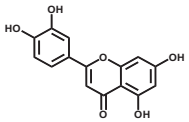
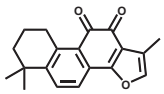
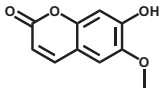
Although a corresponding increase in inhibition of IL-6 production were observed in the MAPK inhibitors treatment group for dose between 0.001 and 1 μM, these inhibitory effects did not reach up as high as luteolin when concentrations exceed to 10 μM (Figure 4B and Supplementary Figure S5). It is also noteworthy that weak inhibitory effect of luteolin against the four MAPK targets were detected around 10 μM (Supplementary Figure S4). Compound concentration above 10 μM was not in consideration according to the cytotoxicity assay (Supplementary Figure S6).

Taken together, multi-weak perturbations of luteolin and tanshinone IIA against the MAPK signaling pathway can potentially decrease the inflammatory response.

Discussion and conclusion

Developing highly selective ligands that interact with individual target proteins has been the dominating drug discovery approach in the past decades [1]. Contrary to highly publicized claims, a highly potent lead compound usually yields a drug candidate that often links to a higher risk of failure during drug development [24]. Meanwhile, analysis of the binding affinities of marketed drugs and natural products indicates that therapeutic efficacy is not necessarily associated with high binding affinity [2]. At the molecular level, weak interactions play critical roles in molecular recognition in biological systems, from the classic example of protein folding to recent discoveries in metabolism, gene regulation and signal transduction [8, 23, 81]. From the point view of network biology, weak interactions contribute toward the robustness and diversity of biological networks, and thus collective weak interactions may have more profound effects on biological systems than a single, strong interaction [29, 82]. Weak interaction can be characterized by

Table 2. Binding free energies and IC_{50} values of the selective inhibitors and the three natural products

Compound	Structure	MEK1 $\Delta G_{bind}/IC_{50}$	ERK2 $\Delta G_{bind}/IC_{50}$	JNK1 $\Delta G_{bind}/IC_{50}$	p38α $\Delta G_{bind}/IC_{50}$
MEK1 inhibitor (PD0325901)		$^{+++}$0.33 nM [73]	$^{+++}$>10 μM [74]	$^{++}$>10 μM [74]	$^{+++}$>10 μM [74]
ERK2 inhibitor (FR180204)		$^{++}$>30 μM [75]	$^{+++}$0.33 μM [75]	$^{++}$—	$^{+++}$10 μM [75]
JNK1 inhibitor (SP600125)		$^{++}$>10 μm [76]	$^{++}$>10 μM [77]	$^{+++}$0.04 μm [77]	$^{++}$>10 μm [77]
p38 inhibitor (SB203580)		$^{+++}$>10 μM [78]	$^{+++}$>10 μM [79]	$^{++}$>100 μM [80]	$^{+++}$0.05 μM [78, 79]
Luteolin		$^{++}$29.6 μM	$^{++}$>200 μM	$^{++}$88.4 μM	$^{++}$91.4 μM
Tanshinone IIA		$^{++}$—	$^{++}$—	$^{++}$—	$^{++}$—
Scopoletin		$^{+}$>200 μM	$^{+}$>200 μM	$^{+}$>200 μM	$^{+}$>200 μM

$^{+}$Very weak affinity: Binding free energy > −10 kcal/mol.
$^{++}$Weak affinity, −25 kcal/mol < Binding free energy < −10 kcal/mol.
$^{+++}$Moderate affinity, −40 kcal/mol < Binding free energy < −25 kcal/mol.
$^{+++}$Strong affinity, Binding free energy < −40 kcal/mol.

the high dissociation rate of the complex. The dissociation rate constant k_{off} may be thus a focal point for medicinal chemistry to consider in terms of designing weak-binding drugs. In our modeling system, we used low inhibition rates of the targets to directly represent the effect of weak-binding drugs rather than the high k_{off} constant in consideration of making the simulation results straighter and reducing the calculation complexity.

Another key issue for designing effective weak-binding drugs may be to find proper drug target combinations within complex cellular networks. Although experimental tools such as HTS hold promise for discovering weak-binding drugs, they are often of low efficiency and time-consuming, and the exponentially increasing number of potential drug target combinations also makes pure experimental tools quickly unfeasible [9]. Appropriate computational models and algorithms, as well as abundant database resources, can effectively reduce the search space for determining promising combinations for experimental evaluation ([Table 3]). Thus, systematic integration of computational tools with experimental strategies can contribute to identifying low affinity hits efficiently and realizing the full potential of weak-binding drugs in different disease phenotypes [10].

In this study, we delineate the efficacy of multi-weak perturbation patterns on different elementary subgraphs to search for optimal drug target combinations in specific pathways. Specifically, we used dynamics simulation to identify biologically significant elementary subgraphs. Such subgraphs seem to capture the essential dynamics of protein circuits, while being, in a sense, insulated from most of the complexity of the proteins themselves. In this way, it would be intriguing to interpret the functionality of these intervention patterns. Interestingly, targeted cancer therapy has provided the practical basis for how the elementary subgraphs in cellular network are perturbed successfully by drug cocktails. For example, co-targeting Akt and mTOR in a STSs, respectively, by MK-2206 and MK-8669 is an effective strategy for basal-like breast cancer [86]. For DPSs, Meng *et al.* [87] reported that combination treatment with MEK and AKT inhibitors is more effective than each drug alone in human non-small-cell lung cancer both *in vitro* and *in vivo*.

Moreover, through assessing the synergistic effect of elementary subgraph under varying parameters, we reveal that the therapeutic effects of targeting these elementary subgraphs can either be parameter dependent or parameter independent. The parameters (K_M, k_{cat}) here depict the underlying kinetic rates and dynamic properties of the signaling pathways, which play key roles in governing cellular functions and coordinating cell actions. Therefore, to achieve desired therapeutic effects, for parameter-independent subgraphs, one only needs to consider

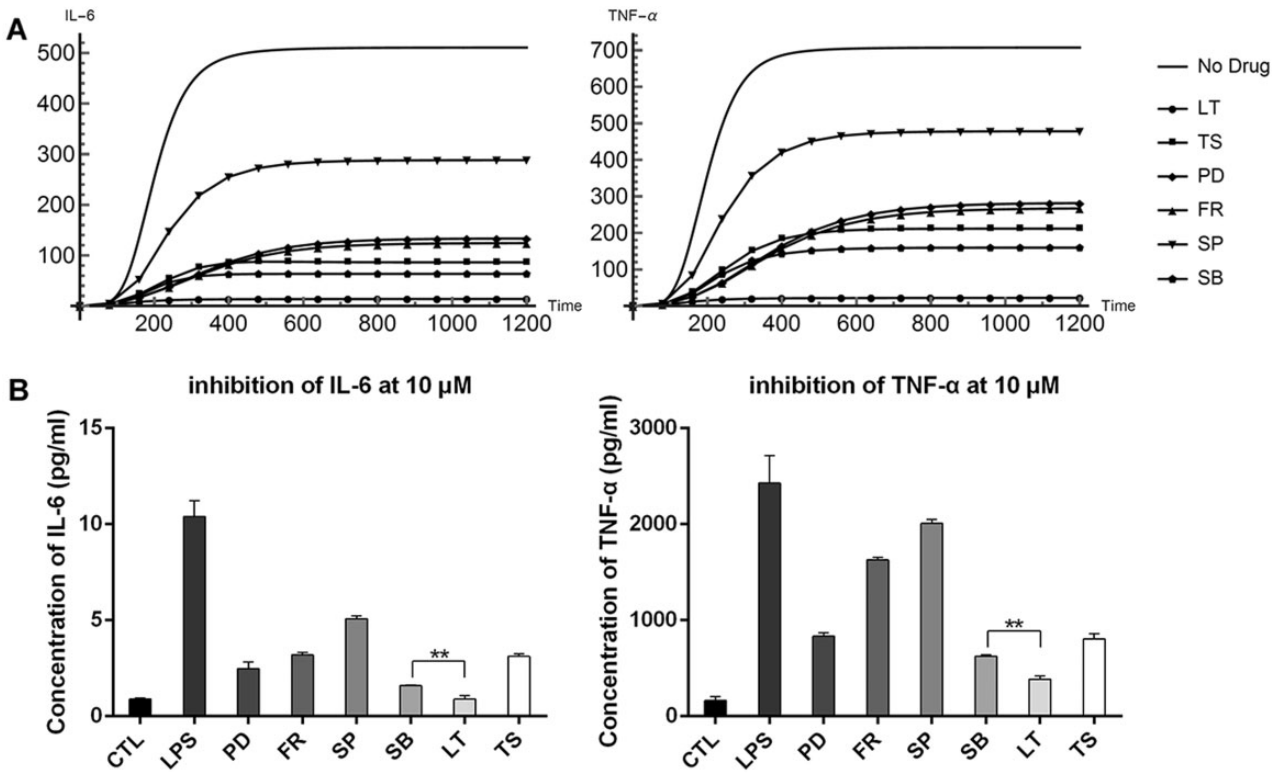


Figure 4. (**A**) The simulated inhibitory effects of natural products [luteolin (LT) and tanshinone IIA (TS)] and selective inhibitors (PD0325901, FR180204, SP600125, SB203580) on IL-6 and TNF-α production at 10 μM. PD: MEKi, FR: ERKi, SP: JNKi, SB: p38i. The inhibition rates of these compounds at 10 μM are inferred from their IC_{50} curves. (**B**) The inhibitory effects of natural products (LT and TS) and selective inhibitors (PD0325901, FR180204, SP600125, SB203580) on IL-6 and TNF-α production in the supernatant of THP-1 cells at 10 μM. CTL: vehicle control, LPS: LPS treatment group. * indicates $p < 0.05$; ** indicates $p < 0.01$ (two-tailed Student's *t*-test). Error bars are standard deviations of measurements.

Table 3. Representative examples of database resources and their application in computational polypharmacology analysis

Databases	URL/availability/developers	Description	Example applications
TCMSP [69]	http://lsp.nwsuaf.edu.cn/tcmsp.php, publicly available, Center of Bioinformatics, Northwest A&F University, Yangling, Shaanxi, China	It consists of 499 Chinese herbs with 29 384 ingredients, including 12 ADME-related properties, known and predicted drug targets and diseases. Compound–target and target–disease networks and tools for network visualization and analysis	Exploring the target space and therapeutic potential of natural products [10], mechanisms of action of traditional Chinese medicines [83]
BindingDB [7]	http://www.bindingdb.org/bind/index.jsp, publicly available, Skaggs School of Pharmacy & Pharmaceutical Sciences, 9500 Gilman Drive, MC 0736, La Jolla, California	Database of measured binding affinities, focusing chiefly on the interactions of protein considered to be drug targets with small, drug-like molecules. Containing 1 155 030 binding data, for 7113 protein targets and 503 693 small molecules.	Prediction of direct drug–target interactions [55]
DrugBank [13]	http://www.drugbank.ca/, publicly available, Department of Computing Science, University of Alberta, Edmonton, AB, Canada	A unique bioinformatics and cheminformatics resource that combines detailed drug data with comprehensive drug target information. Containing 8312 drug entries and 4317 nonredundant proteins.	Facilitating polypharmacology and data integration [11]
The Protein Data Bank [71]	http://www.rcsb.org, publicly available Rutgers, The State University of New Jersey Center for Integrative Proteomics Research 174 Frelinghuysen Rd Piscataway, NJ; San Diego Supercomputer Center (SDSC) and Skaggs School of Pharmacy and Pharmaceutical Sciences; University of California, San Diego (UCSD) 9500 Gilman Drive La Jolla, CA	The single worldwide repository of information about the 3D structures of large biological molecules, including proteins and nucleic acids. Containing 114 741 Biological Macromolecular Structures.	Protein structure prediction [84], protein–ligand docking [85]

the topology relationship between the two targets in the cellular network; while for parameter-dependent subgraphs, one should pay attention to both of the network topology and kinetic properties between the targets.

Applying the elementary subgraphs to the MAPK network results in the efficient identification of the drug target combinations and the effective multi-target weak interventions. We investigated stimulus-specific effects of multi-weak perturbations in the JNK, ERK and p38 MAPK signaling pathways both *in silico* and *in vitro*. Particularly, we found that luteolin, as a multi-targeting kinase inhibitor, shows remarkable inhibitory effects on IL-6 and TNF-α production at 10 μM through its weak inhibitions on four target kinases (ERK, JNK, p38 and MEK), which is a supplement to previous study that found Tumor Progression Locus 2 as a target of luteolin [88]. The multi-target weak intervention pattern provides theoretical and experimental evidence that it can anticipate considerable improvements in the rate of discovery of safe and effective drugs. Together, our work begins to define the opportunities for pharmacological targeting of specific network topologies by weak systematic perturbations to achieve desirable therapeutic effects.

Further, natural products and their combinations commonly interact with multiple drug targets thought to encompass and exceed the currently limited space of targets of Food and Drug Administration-approved drugs, thus holding potential for new types of therapeutic opportunities [10]. Meanwhile, food components having multiple weak targets may have an important role to play in disease prevention and there is scope for the methods described here to be used in discovering which dietary compounds, alone or in combination, play a part in which preventive/therapeutic mechanisms [10]. Weak-binding drugs from these materials have real opportunities for maximum efficiency and, at the same time, they may offer the potential of reduced adverse side effects. Here, we have presented a reliable approach to identify low-affinity compounds. Because this approach can be used to supplement high-affinity target-based drug discovery, we can assume that more therapies could be rediscovered. Therefore, if more scientists develop drugs not only from the compounds that bind tightly to a protein target but also from the small molecules that bind weakly or transiently to multi-targets, new drug discovery will have a bright and promising future.

Key Points

- Weak-binding drugs can have good efficacy and be an important source for new drug discovery.
- Weak-binding drugs can be characterized by their high dissociation rates and transient interactions with their targets.
- Topology structure and dynamics parameters of target network can influence the effects of weak-binding drugs.
- Systematic integration of multiple computational approaches provides a potential strategy for screening new weak-binding drugs.

Supplementary data

Supplementary data are available online at http://bib.oxfordjournals.org/.

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References

1. Hopkins AL. Network pharmacology: the next paradigm in drug discovery. *Nat Chem Biol* 2008;**4**:682–90.
2. Mestres J, Gregori-Puigjane E. Conciliating binding efficiency and polypharmacology. *Trends Pharmacol Sci* 2009;**30**:470–4.
3. Lipton SA. Pathologically-activated therapeutics for neuroprotection: mechanism of NMDA receptor block by memantine and S-nitrosylation. *Curr Drug Targets* 2007;**8**:621–32.
4. Csermely P, Agoston V, Pongor S. The efficiency of multi-target drugs: the network approach might help drug design. *Trends Pharmacol Sci* 2005;**26**:178–82.
5. Tan LT. Bioactive natural products from marine cyanobacteria for drug discovery. *Phytochemistry* 2007;**68**:954–79.
6. Zhu L, Xu X. Selective separation of active inhibitors of epidermal growth factor receptor from Caragana jubata by molecularly imprinted solid-phase extraction. *J Chromatogr A* 2003;**991**:151–8.
7. Liu T, Lin Y, Wen X, *et al*. BindingDB: a web-accessible database of experimentally determined protein-ligand binding affinities. *Nucleic Acids Res* 2007;**35**:D198–201.
8. Ohlson S. Designing transient binding drugs: a new concept for drug discovery. *Drug Discov Today* 2008;**13**:433–9.
9. Tang J, Aittokallio T. Network pharmacology strategies toward multi-target anticancer therapies: from computational models to experimental design principles. *Curr Pharm Des* 2014;**20**:23–36.
10. Kibble M, Saarinen N, Tang J, *et al*. Network pharmacology applications to map the unexplored target space and therapeutic potential of natural products. *Nat Prod Rep* 2015;**32**:1249–66.
11. Barneh F, Jafari M, Mirzaie M. Updates on drug-target network; facilitating polypharmacology and data integration by growth of DrugBank database. *Brief Bioinform* 2015. doi: 10.1093/bib/bbv094.
12. Wishart DS, Knox C, Guo AC, *et al*. DrugBank: a comprehensive resource for in silico drug discovery and exploration. *Nucleic Acids Res* 2006;**34**:D668–72.
13. Law V, Knox C, Djoumbou Y, *et al*. DrugBank 4.0: shedding new light on drug metabolism. *Nucleic Acids Res* 2014;**42**:D1091–7.
14. Yildirim MA, Goh KI, Cusick ME, *et al*. Drug-target network. *Nat Biotechnol* 2007;**25**:1119–26.
15. Nunez S, Venhorst J, Kruse CG. Target-drug interactions: first principles and their application to drug discovery. *Drug Discov Today* 2012;**17**:10–22.
16. Lipton SA. Paradigm shift in neuroprotection by NMDA receptor blockade: memantine and beyond. *Nat Rev Drug Discov*2006;**5**:160–70.
17. Lu H, Tonge PJ. Drug-target residence time: critical information for lead optimization. *Curr Opin Chem Biol* 2010;**14**:467–74.
18. Copeland RA, Pompliano DL, Meek TD. Opinion - Drug-target residence time and its implications for lead optimization. *Nat Rev Drug Discov* 2006;**5**:730–9.
19. Bottegoni G, Favia AD, Recanatini M, *et al*. The role of fragment-based and computational methods in polypharmacology. *Drug Discov Today* 2012;**17**:23–34.

20. Overington JP, Al-Lazikani B, Hopkins AL. Opinion - How many drug targets are there? *Nat Rev Drug Discov* 2006;**5**:993–6.
21. Hopkins AL, Mason JS, Overington JP. Can we rationally design promiscuous drugs? *Curr Opin Struct Biol* 2006;**16**:127–36.
22. Cho DY, Kim YA, Przytycka TM. Chapter 5: Network biology approach to complex diseases. *Plos Comput Biol* 2012;**8**:e1002820.
23. Korcsmaros T, Szalay MS, Csaba B, *et al*. How to design multitarget drugs: target search options in cellular networks. *Expert Opin Drug Discov* 2007;**2**:799–808.
24. Xie L, Xie L, Kinnings SL, *et al*. Novel computational approaches to polypharmacology as a means to define responses to individual drugs. *Annu Rev Pharmacol Toxicol* 2012;**52**:361–79.
25. Lounkine E, Keiser MJ, Whitebread S, *et al*. Large-scale prediction and testing of drug activity on side-effect targets. *Nature* 2012;**486**:361–7.
26. Boran AD, Iyengar R. Systems approaches to polypharmacology and drug discovery. *Curr Opin Drug Discov Devel* 2010; **13**:297–309.
27. Rosini M. Polypharmacology: the rise of multitarget drugs over combination therapies. *Future Med Chem* 2014;**6**:485–7.
28. Chen B, Wild D, Guha R. PubChem as a source of polypharmacology. *J Chem Inf Model* 2009;**49**:2044–55.
29. Csermely P. Strong links are important, but weak links stabilize them. *Trends Biochem Sci* 2004;**29**:331–4.
30. Gribbon P, Sewing A. High-throughput drug discovery: what can we exulect from HTS? *Drug Discov Today* 2005;**10**:17–22.
31. Wang Y, Xing J, Xu Y, *et al*. In silico ADME/T modelling for rational drug design. Q *Rev Biophys* 2015;**48**:488–515.
32. Macarron R, Banks MN, Bojanic D, *et al*. Impact of high-throughput screening in biomedical research. *Nat Rev Drug Discov* 2011;**10**:188–95.
33. Vaynberg J, Qin J. Weak protein-protein interactions as probed by NMR spectroscopy. *Trends Biotechnol* 2006;**24**:22–7.
34. Ozbal CC, LaMarr WA, Linton JR, *et al*. High throughput screening via mass spectrometry: a case study using acetylcholinesterase. *Assay Drug Dev Technol* 2004;**2**:373–81.
35. Nienaber VL, Richardson PL, Klighofer V, *et al*. Discovering novel ligands for macromolecules using X-ray crystallographic screening. *Nat Biotechnol* 2000;**18**:1105–8.
36. Duong-Thi MD, Meiby E, Bergstrom M, *et al*. Weak affinity chromatography as a new approach for fragment screening in drug discovery. *Anal Biochem* 2011;**414**:138–46.
37. Rudnev AV, Aleksenko SS, Semenova O, *et al*. Determination of binding constants and stoichiometries for platinum anticancer drugs and serum transport proteins by capillary electrophoresis using the Hummel-Dreyer method. *J Sep Sci* 2005;**28**:121–7.
38. Rich RL, Myszka DG. Higher-throughput, label-free, real-time molecular interaction analysis. *Anal Biochem* 2007;**361**:1–6.
39. Christopher JA, Brown J, Dore AS, *et al*. Biophysical fragment screening of the beta(1)-adrenergic receptor: identification of high affinity arylpiperazine leads using structure-based drug design. *J Med Chem* 2013;**56**:3446–55.
40. Barabasi AL, Gulbahce N, Loscalzo J. Network medicine: a network-based approach to human disease. *Nat Rev Genet* 2011;**12**:56–68.
41. Araujo RP, Liotta LA, Petricoin EF. Proteins, drug targets and the mechanisms they control: the simple truth about complex networks. *Nat Rev Drug Discov* 2007;**6**:871–80.
42. Yang K, Bai HJ, Qi OY, *et al*. Finding multiple target optimal intervention in disease-related molecular network. *Mol Syst Biol* 2008;**4**:228.
43. Bianconi F, Baldelli E, Ludovini V, *et al*. Computational model of EGFR and IGF1R pathways in lung cancer: a systems biology approach for translational oncology. *Biotechnol Adv* 2012;**30**:142–53.
44. Fujioka A, Terai K, Itoh RE, *et al*. Dynamics of the Ras/ERK MAPK cascade as monitored by fluorescent probes. *J Biol Chem* 2006;**281**:8917–26.
45. Li P, Fu Y, Ru J, *et al*. Insights from systems pharmacology into cardiovascular drug discovery and therapy. *BMC Syst Biol* 2014;**8**:141
46. Hwang WC, Zhang A, Ramanathan M. Identification of information flow-modulating drug targets: a novel bridging paradigm for drug discovery. *Clin Pharmacol Ther* 2008;**84**:563–72.
47. Courtney KD, Corcoran RB, Engelman JA. The PI3K pathway as drug target in human cancer. *J Clin Oncol* 2010;**28**:1075–83.
48. Cheng JQ, Lindsley CW, Cheng GZ, *et al*. The Akt/PKB pathway: molecular target for cancer drug discovery. *Oncogene* 2005;**24**:7482–92.
49. Sykiotis GP, Papavassiliou AG. Serine phosphorylation of insulin receptor substrate-1: a novel target for the reversal of insulin resistance. *Mol Endocrinol* 2001;**15**:1864–9.
50. Xie L, Evangelidis T, Xie L, *et al*. Drug discovery using chemical systems biology: weak inhibition of multiple kinases may contribute to the anti-cancer effect of nelfinavir. *Plos Comput Biol* 2011;**7**:e1002037.
51. Kitchen DB, Decornez H, Furr JR, *et al*. Docking and scoring in virtual screening for drug discovery: methods and applications. *Nat Rev Drug Discov* 2004;**3**:935–49.
52. Kollman PA, Massova I, Reyes C, *et al*. Calculating structures and free energies of complex molecules: combining molecular mechanics and continuum models. *Acc Chem Res* 2000;**33**:889–97.
53. Durrant JD, McCammon JA. Molecular dynamics simulations and drug discovery. *BMC Biol* 2011;**9**:71.
54. Yu H, Chen J, Xu X, *et al*. A systematic prediction of multiple drug-target interactions from chemical, genomic, and pharmacological data. *PLoS One* 2012;**7**:e37608.
55. Zheng C, Guo Z, Huang C, *et al*. Large-scale direct targeting for drug repositioning and discovery. *Sci Rep* 2015;**5**:11970.
56. Yin N, Ma W, Pei J, *et al*. Synergistic and antagonistic drug combinations depend on network topology. *PLoS One* 2014;**9**:e93960.
57. Ma WZ, Trusina A, El-Samad H, *et al*. Defining network topologies that can achieve biochemical adaptation. *Cell* 2009;**138**:760–73.
58. Fitzgerald JB, Schoeberl B, Nielsen UB, *et al*. Systems biology and combination therapy in the quest for clinical efficacy. *Nat Chem Biol* 2006;**2**:458–66.
59. Behar M, Barken D, Werner SL, *et al*. The dynamics of signaling as a pharmacological target. *Cell* 2013;**155**:448–61.
60. Shoval O, Alon U. SnapShot: network motifs. *Cell* 2010;**143**:326–U158.
61. Mangan S, Alon U. Structure and function of the feed-forward loop network motif. *Proc Natl Acad Sci USA* 2003;**100**:11980–5.
62. Goentoro L, Shoval O, Kirschner MW, *et al*. The incoherent feedforward loop can provide fold-change detection in gene regulation. *Mol Cell* 2009;**36**:894–9.
63. Hart Y, Alon U. The utility of paradoxical components in biological circuits. *Mol Cell* 2013;**49**:213–21.
64. Sabio G, Davis RJ. TNF and MAP kinase signalling pathways. *Semin Immunol* 2014;**26**:237–45.
65. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002;**298**:1911–12.

66. Ventura JJ, Kennedy NJ, Lamb JA, *et al.* c-jun NH2-terminal kinase is essential for the regulation of AP-1 by tumor necrosis factor. *Mol Cell Biol* 2003;**23**:2871–82.
67. Lee JC, Laydon JT, Mcdonnell PC, *et al.* A protein-kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 1994;**372**:739–46.
68. Tomida T, Takekawa M, Saito H. Oscillation of p38 activity controls efficient pro-inflammatory gene expression. *Nat Commun* 2015;**6**:8350.
69. Ru JL, Li P, Wang JN, *et al.* TCMSP: a database of systems pharmacology for drug discovery from herbal medicines. *J Cheminform* 2014;**6**:13.
70. Wang Y, Zheng C, Huang C, *et al.* Systems pharmacology dissecting holistic medicine for treatment of complex diseases: an example using cardiocerebrovascular diseases treated by TCM. *Evid Based Complement Alternat Med* 2015;**2015**:980190.
71. Berman HM, Westbrook J, Feng Z, *et al.* The protein data bank. *Nucleic Acids Res* 2000;**28**:235–42.
72. Zhang H, Yao Y, Yang H, *et al.* Molecular dynamics and free energy studies on the carboxypeptidases complexed with peptide/small molecular inhibitor: mechanism for drug resistance. *Insect Biochem Mol Biol* 2012;**42**:583–95.
73. Barrett SD, Bridges AJ, Dudley DT, *et al.* The discovery of the benzhydroxamate MEK inhibitors CI-1040 and PD 0325901. *Bioorg Med Chem Lett* 2008;**18**:6501–4.
74. Bain J, Plater L, Elliott M, *et al.* The selectivity of protein kinase inhibitors: a further update. *Biochem J* 2007;**408**:297–315.
75. Ohori M, Kinoshita T, Okubo M, *et al.* Identification of a selective ERK inhibitor and structural determination of the inhibitor-ERK2 complex. *Biochem Biophys Res Commun* 2005;**336**:357–63.
76. Bain J, McLauchlan H, Elliott M, *et al.* The specificities of protein kinase inhibitors: an update. *Biochem J* 2003;**371**:199–204.
77. Bennett BL, Sasaki DT, Murray BW, *et al.* SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci USA* 2001;**98**:13681–6.
78. Eyers PA, Craxton M, Morrice N, *et al.* Conversion of SB 203580-insensitive MBP kinase family members to drug-sensitive forms by a single amino-acid substitution. *Chem Biol* 1998;**5**:321–8.
79. Davies SP, Reddy H, Caivano M, *et al.* Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 2000;**351**:95–105.
80. Cuenda A, Rouse J, Doza YN, *et al.* Sb-203580 is a specific inhibitor of a map kinase homolog which is stimulated by cellular stresses and interleukin-1. *FEBS Lett* 1995;**364**:229–33.
81. Tanay A. Extensive low-affinity transcriptional interactions in the yeast genome. *Genome Res* 2006;**16**:962–72.
82. Agoston V, Csermely P, Pongor S. Multiple weak hits confuse complex systems: a transcriptional regulatory network as an example. *Physical Rev E Stat Nonlin Soft Matter Phys* 2005;**71**:051909.
83. Huang C, Zheng CL, Li Y, *et al.* Systems pharmacology in drug discovery and therapeutic insight for herbal medicines. *Brief Bioinform* 2014;**15**:710–33.
84. Kelley LA, Sternberg MJE. Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* 2009;**4**:363–71.
85. Verdonk ML, Cole JC, Hartshorn MJ, *et al.* Improved protein-ligand docking using GOLD. *Proteins* 2003;**52**:609–23.
86. Xu SG, Li SQ, Guo ZF, *et al.* Combined targeting of mTOR and AKT is an effective strategy for basal-like breast cancer in patient-derived xenograft models. *Mol Cancer Ther* 2013;**12**:1665–75.
87. Meng JR, Dai BB, Fang BL, *et al.* Combination treatment with MEK and AKT inhibitors is more effective than each drug alone in human non-small cell lung cancer in vitro and in vivo. *PLoS One* 2010;**5**:e14124.
88. Kim JE, Son JE, Jang YJ, *et al.* Luteolin, a novel natural inhibitor of tumor progression locus 2 serine/threonine kinase, inhibits tumor necrosis factor-alpha-induced cyclooxygenase-2 expression in JB6 mouse epidermis cells. *J Pharmacol Exp Ther* 2011;**338**:1013–22.