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REVIEW

Screening-based approaches to identify small molecules that inhibit protein–protein interactions

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ABSTRACT

Introduction: Protein–protein interactions (PPIs) are very attractive targets for drug development as they play important roles in regulating many aspects of pathophysiologies. It has recently been revealed that the functionally important region of most PPIs is small enough to be modulated by small molecules. Thus, many studies in this field have achieved amazing progress, together with diverse and advanced screening technologies.

Areas covered: This article presents screening technologies to identify small molecule inhibitors of PPIs in addition to discussing the suitability of PPIs as molecular targets. The phases in the processes of selecting compounds are discussed and appropriate steps are proposed, including methodologies to test binding affinity, kinetics, structural analysis, and cellular function.

Expert opinion: Targeting PPIs is still a challenging approach in drug development and relatively few small molecules have reached clinical development. Potential candidates should be assessed and optimized by properly using the multiple assay systems to develop ideal small molecule drugs. Although there remain some barriers to be overcome, small molecule inhibitors of PPIs are fascinating and first-in-class as therapeutic agents to treat various diseases.

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KEYWORDS
High-throughput screening; hot spot; small molecule; protein–protein interaction

1. Introduction

Most biological processes and disease states are regulated by multi-protein complexes which are mediated by specific protein–protein interactions (PPIs) [1–4]. Therefore, the modulation of PPIs is a crucial target for drug discovery. On the basis of active research into the structure and function of proteins, many attempts have been made to identify druggable PPI inhibitors [5–10].

Therapeutic antibodies have received attention as the most dynamic and effective candidates for PPI inhibition [11–13]. Because antibodies are specific for their molecular targets and stable in human serum, their presence in the drug market has been rapidly growing. However, antibodies also have some drawbacks, such as high cost, difficulty in manufacturing, and the lack of oral bioavailability. Furthermore, the inability of antibodies to penetrate the cell membrane limits their usage to controlling cell-surface proteins, rendering them unable to target cytoplasmic and nuclear components.

Considering the broad and flat area of PPI interfaces, peptides are ideal candidates because they can closely mimic the subtle features of PPIs [14–16]. In addition, they can adopt diverse structures, and it is easy to improve peptide properties such as stability, binding affinity, and bioavailability because they are convenient to synthesize and modify. Moreover, they are relatively safe for human applications because of their complete biocompatibility [17]. However, peptides present some weaknesses in development as therapeutic agents. They have poor chemical and physical stability against proteolytic degradation, as well as a short circulating half-life in the plasma. Moreover, they are potentially immunogenic and their general hydrophilicity gives them a poor capacity to pass through physiological barriers [18]. The majority of natural alternatives targeting PPIs, such as recombinant proteins, antibodies, and peptides, are also limited by the necessity of administration by injection and the cost compared to small molecules. Recent studies have shown that small molecules, which can be defined as low molecular weight organic compounds with fewer than 900 Da, are potential candidates for PPI inhibition [19–24]. These molecules have many advantages for regulating biological processes. They are easier and cheaper to manufacture than protein-based drugs. Additionally, small molecules can be administered orally, in the form of a tablet or capsule. Therefore, small molecules are favorable in preclinical and clinical development, have a low mass production cost, and already account for the majority of drugs in the global marketplace. In this review, we first explain the characteristics of PPI interfaces, including how such interfaces can be inhibited by small molecules. We then mention several screening methodologies and strategies to identify small molecules that modulate PPIs. In addition, we also introduce several newly developed screening technologies. Finally, we describe the current status of the development of small molecule inhibitors targeting PPIs, as well as obstacles to development and future prospects.

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Article highlights

- Protein–protein interactions (PPIs) are prospective targets for development of therapeutic drugs because many essential biological processes are mediated by interactions between proteins.
- Although PPI interfaces are large and flat, most binding energy is conferred by regions small enough to cover with small molecules, referred to as the 'hot spot'.
- Small molecules can modulate PPIs by direct binding to the interface of one binding partner.
- For development of small molecule drugs applicable to the clinic, a series of processes, including determination of the binding kinetics and the complex structure, as well as the characterization of the molecule's functions are needed to filter out artifacts and validate the efficacy after high-throughput screening
- Optimization of a compound's activity and druggablity is strengthened by analogue synthesis using structure-based drug design.
- Small molecule inhibitors of PPI will occupy a large portion of the pharmaceutical industry in the future.

This box summarizes key points contained in the article.

2. Protein–protein interfaces as targets of small molecules

PPI interfaces have a great variety of sizes, shapes, binding affinities, and assembly dynamics [25]. PPIs are usually classified into three groups, depending on whether the interactions are between two protein domains (domain–domain), a domain and a relatively short peptide (domain–peptide), or two peptide stretches (peptide–peptide). Typically, PPI contact surfaces are large (with a buried area of approximately 1500–3000 $Å^2$ per side) and include a number of polar and hydrophobic interactions. PPI interfaces are also generally flat and ill defined, with variable contact points and a lack of deep and well-defined binding cavities. These features are the greatest obstacles to modulating PPIs using small molecules [26–28].

However, in recent years, many studies have illustrated that PPIs often do not require the entire protein-binding surface. Mutational analyses of PPI interfaces, such as alanine scanning, verified that binding affinity is not evenly distributed across the surfaces, but rather that most of the Gibbs binding energy is conferred by a small patch of amino-acid residues, often near the center of the interface, referred to as a 'hot spot' [29–34]. Most hot spots are compact enough in size to be covered by a small molecule.

These features support the possibility that small molecules can effectively inhibit PPIs by targeting specific hot spots on the PPI interface. Over the last decade, small molecular approaches to control PPIs have improved drastically as screening technologies have advanced. One of these successes is the development of tirofiban, an αIIbβ3 antagonist, as a drug for the treatment of acute coronary syndromes [35–37]. Many small molecule drugs are currently being developed, and several are presently undergoing clinical trials. Table 1 shows examples of small molecules modulating PPIs that are under clinical developmental stages. The small molecules that inhibit the Ras–SOS1 complex, the Raf dimer, the YAP/TAZ–TEAD interaction, the LEDGF/p75–integrase interaction, or the CXXC5–Dishevelled interaction are the newly identified interesting PPI inhibitors. For example, DCAI (4,6-dichloro-2-methyl-3-aminoethyl-indole) represents a significant advancement in inhibitors of Ras oncogene and indicates anticancer effects by blocking the Ras–SOS1 interaction [38,39]. Verteporfin that suppresses the YAP-TEAD complex shows the effectiveness for the treatment of breast cancer, ovarian cancer, prostate carcinoma, and pancreatic ductal adenocarcinoma [40,41]. LEDGINs that disrupt the LEDGF/p75–integrase interaction and thereby inhibit HIV replication are under development for clinical validation [42,43]. KY-02327 inhibiting the CXXC5–Dishevelled interaction is a potential drug candidate that enhances anabolic bone formation via activation of the Wnt/β-catenin pathway [44,45]. It is expected that more diverse PPIs and their modulators, which improve pharmacological value, will be discovered through the development of new technologies.

3. High-throughput screening

In the past few years, a variety of screening technologies have been developed for drug discovery [46,47]. In particular, a number of high-throughput screening (HTS) methods compatible with PPI inhibitor screening have greatly progressed [48–51]. These methods are useful for rapid and simple identification of initial hits from libraries containing thousands or millions of

Table 1. Small molecule inhibitors against protein–protein interactions in clinical development.

PPI	Compound	Clinical stage	Target biology	ClinicalTrials.gov identifier
MDM2–p53	AMG232	Phase I/II	Cancer	NCT02110355
	ALRN-6924	Phase I	Cancer	NCT02264613
	CGM097	Phase I	Cancer	NCT01760525
	DS-3032b	Phase I	Cancer	NCT01877382
	Idasanutlin	Phase III	Cancer	NCT02545283
	MK-8242	Phase I	Cancer	NCT01463696
	RG-7112	Phase I	Cancer	NCT00623870
BCL2/BCL-X_L–BAX/BAK	Navitoclax	Phase II	Cancer	NCT01557777
	Venetoclax	Phase I	Cancer	NCT01682616
XIAP–Caspase9	AEG40826	Phase I	Cancer	NCT00708006
	ASTX-660	Phase I/II	Cancer	NCT02503423
	AT-406	Phase II	Cancer	NCT02022098
	Birinapant	Phase I/II	Cancer	NCT02587962
	CUDC-427	Phase I	Cancer	NCT01908413
	LCL-161	Phase II	Cancer	NCT01955434
Fibrinogen–αIIbβ3	Tirofiban	Approved	Cardiovascular	NCT01109134

Sources taken from https://clinicaltrials.gov/.

compounds. Typically, these HTS methods use fluorescence or chemiluminescence to detect a change in signal intensity.

3.1. Fluorescence polarization

Fluorescence polarization (FP) is one method that can be used to quantitatively measure the strength of the PPIs [52,53]. FP is based on the relationship between the polarization of a fluorophore and the modulation of its anisotropy value [54]. Linearly polarized light, which is produced by passing light through an excitation polarizing filter, excites a fluorophore and then becomes partially depolarized by molecular rotation of the fluorophore. The degree of polarization, either parallel or perpendicular to the excitation light plane, can be monitored by an emission polarizer. The emission light intensity is determined by the size of fluorophore. When using this method to investigate PPI modulators, a fluorescently labeled peptide that includes the 'hot spots' of one of the protein partners is generally used as the fluorophore. When a fluorescently labeled peptide is present freely in solution, it quickly rotates and depolarizes the initially polarized light, resulting in low FP signal (Figure 1). The interaction with its protein partner causes decreased depolarization because the peptide rotates more slowly, resulting in high FP signal. If a small molecule that inhibits the interaction between the protein and the fluorescently labeled peptide is added, it will release the peptide and subsequently increase depolarization, resulting in reduced FP signal. This method has been already widely used for HTS because it is affordably and homogeneous (i.e. a simple mix-and-read procedure without separation or washing steps to introduce variability) [55,56]. However, this assay can produce autofluorescence and light scattering, which disturbs detection of the FP signal. In addition, testing a high concentration of small molecules can induce anomalous polarization through nonspecific interactions between the fluorescent probe and small molecule aggregates. In particular, hydrophobic small molecules often form micelle-like particles [57]. Thus, it is difficult to distinguish false positives when using high concentrations of small molecules because of anomalous high FP signals.

3.2. Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) is a method measuring energy transfer between two fluorophores that are sensitive to light [58,59]. When a donor fluorophore that is electronically excited is located close to an acceptor molecule, energy is transferred and absorbed into the acceptor fluorophore. The energy transfer takes place only when the distance between the donor and the acceptor is less than approximately 10 nm, depending on the type of acceptor and arrangements of the fluorescent molecules. FRET is extremely sensitive to small changes in distance [60]. To identify the PPIs, fluorescent labels, typically cyan fluorescent protein and yellow fluorescent protein, are attached to each partner [61]. The interaction between the two fluorescently labeled proteins is assessed by detecting the level of energy transfer. Incubation with a small molecule inhibiting the PPI will block the energy transfer, increasing fluorescence (Figure 2). This assay can even be used to monitor instantaneous, real-time PPIs in live cells by measuring FRET signal using fluorescence microscopy or FACS, allowing detection of transient interactions. This assay is also useful for detecting the dynamic equilibrium of an interaction, such as the formation and dissociation of a complex, because the interaction of the fluorophores is reversible. However, this assay can be less sensitive than other fluorescence-based assays because close proximity of the fluorophore is required for energy transfer and thus, FRET signal can be obstructed by strong background autofluorescence in the fluorophore itself. The background signal must be subtracted to measure the changes in fluorescence intensity that are produced by the presence or absence of energy transfer. Finally, it is difficult to distinguish weak interactions from the background signal. Also, FRET signal can be lost by photobleaching over time depending on the fluorophores used.

3.3. Amplified luminescent proximity homogeneous assay screen

The amplified luminescent proximity homogeneous assay screen (ALPHAScreen) is a proximity-based assay using donor and acceptor beads, which are coated with a layer of hydrogel

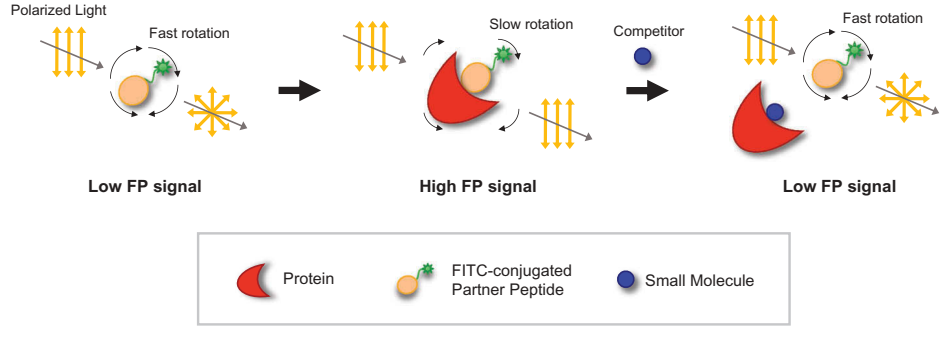

Figure 1. A scheme of fluorescence polarization (FP)-based competitive binding assay. The FITC-labeled peptide containing the 'hot spot' residues for the binding of a protein produces a low FP value by its strong depolarization of the light. Addition of its partner protein induces the protein–peptide complexes formation, resulting in a high FP value by reduction of the depolarization. A small molecule competing for binding of the partner protein results in release of the FITC-labeled peptide and reduces the FP signal because of increased depolarization.

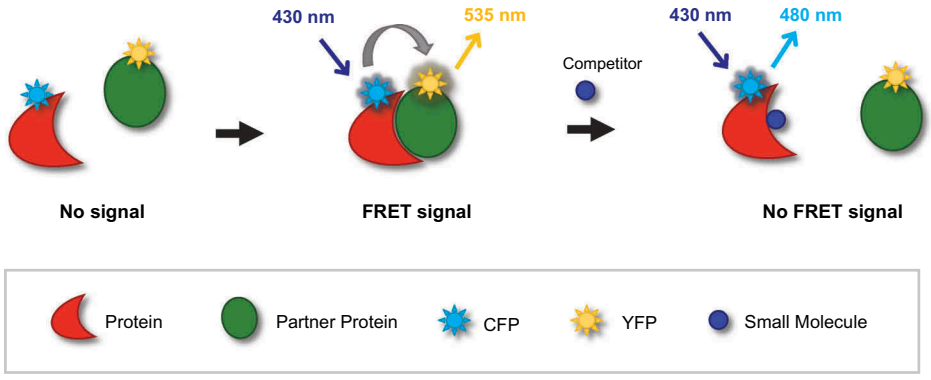

Figure 2. A scheme of fluorescence resonance energy transfer (FRET)-based competitive binding assay. A protein is fused with CFP and the partner protein is fused with YFP. When these two fusion proteins interact, FRET occurs between the donor and acceptor fluorophores due to their proximity. Therefore, excitation at the absorbance wavelength of CFP (430 nm) causes an emission at the YFP wavelength (535 nm). A small molecule that binds to a fusion protein abolishes the FRET signal by dissociating the two fusion proteins, resulting in an emission at the CFP wavelength (480 nm). CFP, cyan fluorescent protein, YFP, yellow fluorescent protein.

offering functional groups for bioconjugation, to detect the binding efficiency of two interacting proteins. The donor beads contain a photosensitizing phthalocyanine that releases an excited and reactive singlet oxygen upon laser irradiation at 680 nm. This singlet oxygen can diffuse in solution within a distance of approximately 200 nm. When acceptor beads are within this distance, the energy in the singlet oxygen is transferred to the acceptor beads, which contain chemiluminescer or fluorescencer, resulting in emission of a signal at 520–620 nm. To apply this principal to study PPIs, one interacting protein is linked to the donor beads and the other to the acceptor beads (Figure 3). If the two proteins interact, excitation of the donor bead will generate a signal. Binding of a small molecule inhibitor to one of the two partner proteins would then disrupt the PPI and the transfer of the singlet oxygen, resulting in loss of the emission signal. This assay is appropriate to detect the interaction between two proteins at a longer distance than the FRET assay. It can be conveniently performed in a microplate format [62–64]. Other advantages of this assay system include its use of nonradioactive beads and the lack of filtration or washing steps. However, this assay requires a specialized reader, because standard fluorimeters and luminometers are not suitable to detect the signal. In addition, the signal is temperature sensitive; so, the assay requires equilibration of the plate at room temperature during signal detection. Furthermore, a large proportion of screening hits could be false positives because the signal is produced by the result of chemical reactions.

3.4. *In vitro* binding assay

An *in vitro* binding assay recently developed by Kim et al. [45] is based on the competitive binding of a small molecule. In this method, one partner protein is substituted for a synthetic peptide that contains its 'hot spot' epitopes and is labeled with fluorescein isothiocyanate (FITC). The interacting domain of the partner protein is immobilized to the polystyrene surface of a microtiter plate. When the FITC-conjugated peptide is applied, it binds to the immobilized protein (Figure 4). If a candidate small molecule is added, it competes with the peptide for binding with the protein domain. Unbound molecules are then washed away. Binding of the peptide can be detected by measurement of the FITC signal, whereas binding of the small molecule displaces the peptide from the partner protein and leads to lower FITC signal. This *in vitro* binding assay is highly useful for HTS because it allows the easy and rapid screening of a chemical library using a microplate reader. In addition, this method is inexpensive and efficient because it uses fluorescent labeling without any radioisotopes. In this method, the feasibility of the PPI as a drug target can be validated before the chemical library screening by testing the functionality of the peptide, which is a substitute for one

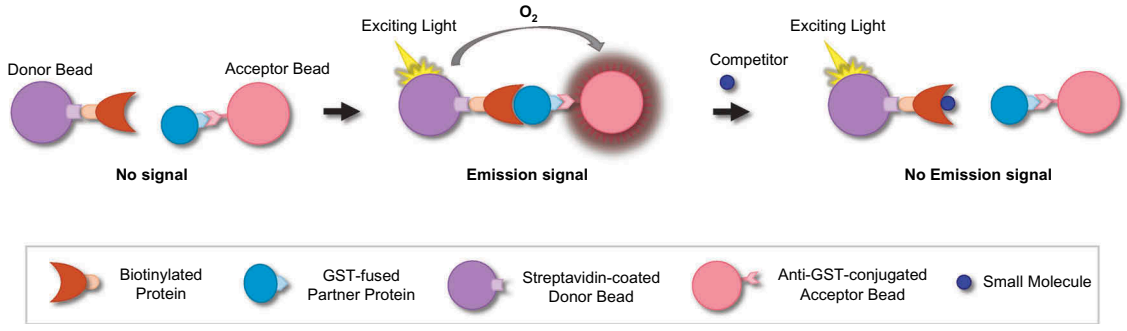

Figure 3. A scheme of amplified luminescent proximity homogeneous assay screen (ALPHA Screen)-based competitive binding assay. A biotinylated protein is attached to Streptavidin-coated donor beads, and a GST-fused partner protein is attached to anti-GST-conjugated acceptor beads. The interaction between the two proteins brings the donor and acceptor beads into close proximately. Reactive oxygen, which is generated by irradiation of the donor beads, produces an emission signal (such as luminescence or fluorescence) in the acceptor bead. A small molecule inhibitor can block the protein–protein interaction, resulting in a decrease in the emission signal.

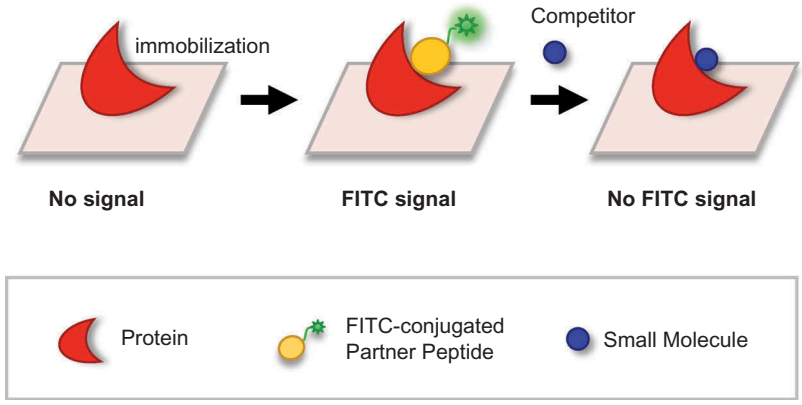

Figure 4. A scheme of *in vitro* binding assay. A protein (or a single domain) involved in a PPI is attached to the polystyrene-coated surface of each well of microplate. Addition of a FITC-conjugated peptide that contains the hot spot residues of the partner protein results in complex formation between the immobilized protein and the FITC-conjugated peptide, and thus a FITC signal. A competitive small molecule induces dissociation of the peptide from the surface of plate, and after washing out unbound peptide, results in a decrease in FITC signal.

partner of the PPI. However, in this system, it is necessary to identify the PPI in detail, including characterization of epitope that is used to design a FITC-tagged peptide and identification of the domain or site of partner protein that binds the peptide, unless intact whole protein is attached to the surface. This assay has been successfully used for the HTS of small molecules blocking the Dvl–CXXC5 interaction, which are potential drug candidates for treatment of osteoporosis [44].

4. Hit validation

Although HTS systems help to identify hits quickly, they often result in positive hits that are artifacts because each assay system can lead to particular biased outcomes [65]. Therefore, candidates obtained from initial screening need to be validated through further analyses to rule out false positives by using alternative and more accurate analyses [66]. Further characterization of the small molecule–target protein interaction should be performed by determination of the binding kinetics and the complex structure, as well as the characterization of the molecule's functions. These steps are important to save time and money during the long, expensive process of drug development (Figure 5).

4.1. Binding kinetics

4.1.1. Surface plasmon resonance

Surface plasmon resonance is a technique for measuring changes in refractive index in real time [67–69]. In this method, a protein of interest is immobilized on a sensor surface composed of a thin gold film coated with carboxymethyl dextran. Polarized light can excite the surface plasmons on the gold film. Injection of a small molecule that can bind to the immobilized protein causes the accumulation of small molecules on the sensor surface. This then increases the refractive index of polarized light, changing the surface plasmon signal [70,71]. This method is advantageous because it allows measurement of the binding affinities as well as the association and dissociation constants, without any labeling.

4.1.2. Bio-layer interferometry

Bio-layer interferometry (BLI) is a newly developed technology based on the optical interferometry, and it allows to detect PPIs in a real time [72]. Similar to surface plasmon resonance, a protein is immobilized to the biosensor surface. BLI analyzes the interference pattern of the reflected light from each of two surfaces that one is the protein binding surface and the other is its interspace. When a small molecule candidate binds to the immobilized protein, optical thickness of the sensor layer is increased and the interference pattern is shifted. This method is a fluidics-free system to analyze molecular interactions. Therefore, it is more useful than surface plasmon resonance in terms of time and costs. Many samples can be analyzed at a time, and the samples can be reused.

4.1.3. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) allows the identification of thermodynamic parameters of PPIs in solution by measuring temperature changes due to heat that is absorbed or released as proteins interact [73–75]. Therefore, this method allows the determination of binding constants, changes in enthalpy and entropy, and the underlying stoichiometry of the PPI. This method measures the temperature difference between two cells using heat-sensing devices. One cell is the reference cell, acting as a control, and the other is sample cell containing a protein of interest. When the partner protein is injected into the sample cell and the PPI occurs, the temperature increases, and the temperature difference between two cells is detected. This method is advantageous in that it determines the kinetics for the molecular interactions in their native states, without any labeling or immobilization of the proteins. However, this method requires a relatively large amount of protein. Therefore, this assay system is of limited use for cases in which preparation of proteins is difficult.

4.1.4. Microscale thermophoresis

Microscale thermophoresis (MST) that was recently developed by NanoTemper technologies allows quantitative analysis of PPIs by measuring the directed movement of proteins in microscopic temperature gradients [76]. In this

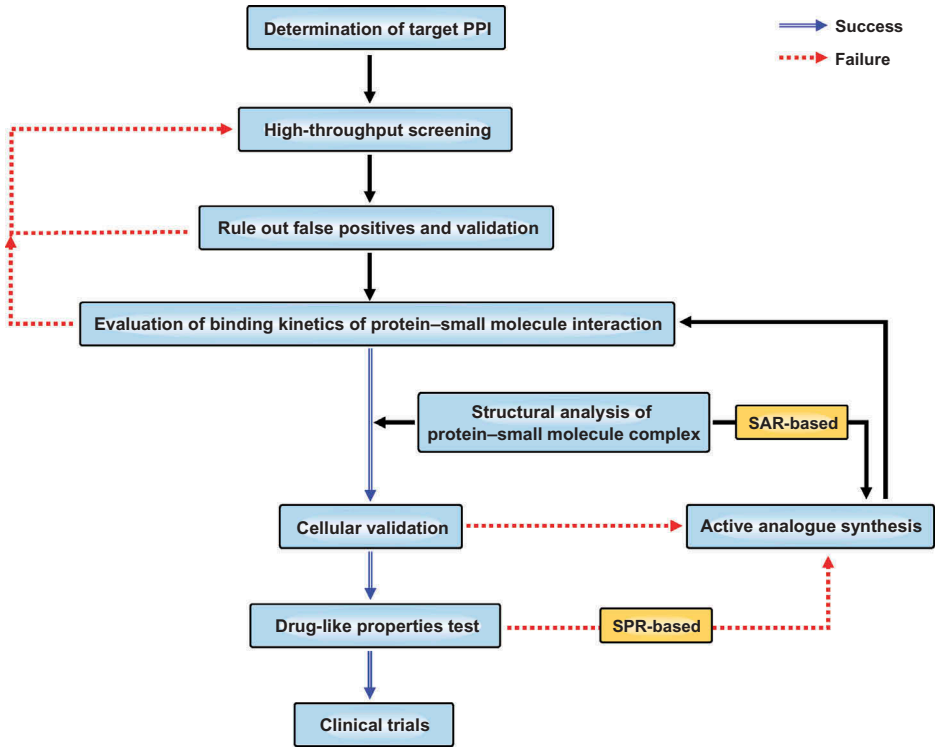

Figure 5. Overview of the drug discovery process for small molecules against protein–protein interactions (PPIs). The determination of a proper PPI target is the first step for drug development. Detailed characterization of the PPI is preferred for better designing and selection of a screening system. The high-throughput screening and validations are undertaken to rule out false positives and to identify initial lead compounds with the potential for PPI modulation. The initial hits are further evaluted through diverse methodologies including measurement of binding kinetics and functional characterization by cellular system. Structural analysis not only supports the binding ability of the hit compounds but also helps SAR-based analogue synthesis. If the final compound selected is inappropriate for clinical development, this lead compound can be optimized by SPR-based chemical synthesis of mimetics and subsequent characterization to improve its drug-like properties. SAR, structure-activity relationship, SPR, structure-property relationship.

method, the samples are placed in thin capillaries in free solution. When PPIs occur, an infrared laser induces local heating, and then molecular mobility in the temperature gradient is detected via fluorescence. Unlike ITC, MST is highly sensitive in detecting all types of binding-induced changes of molecular properties such as size, charge, and hydration shell, with the usage of low amount of samples. Although MST requires one binding partner to be fluorescently labeled, label-free condition is possible in case of proteins that retain intrinsic UV-fluorescence. Furthermore, MST is highly flexible in the types of buffers and additives. Therefore, MST has an advantage in the measurement of PPIs in native conditions even in the context of a complex cell lysate.

4.2. Structural analysis

4.2.1. Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) has been used to determine the physical and chemical properties of atoms or molecules by analyzing the magnetic properties of atomic nuclei, which have electrical charge and spin. To identify an interaction between a small molecule and a protein, usage of NMR is restricted by several limitations, including the requirements of isotope labeling and a high concentration of purified, soluble protein. An expensive instrument is also required for generation of high quality data. Progressive NMR-based techniques have been designed for the confirmation of PPI inhibitors [77,78]. These techniques include chemical shift perturbation (CSP), tranferred nuclear Overhauser effect (NOE), interligand NOE, target immobilized NMR screening, ^{19}F-NMR screening, and saturation transfer difference. To demonstrate a protein–small molecule interaction, the CSP is primarily used to provide information regarding the binding residues on the partner protein. The specific peaks of isotope-labeled proteins are shifted by small molecule binding, and the magnitude of the shift is analyzed to map the precise binding site of the small molecule as well as to calculate the affinity constant. This NMR analysis is especially useful for the structure–function analyses of the relationship between compounds and target using proteins containing point mutations in the amino acid residues required for compound binding. This will be useful for designing analogs in searching compounds that may be more effective [79–82].

4.2.2. X-ray crystallography

X-ray crystallography is a method used to determine three-dimensional molecular structure [83–86]. Accurate positions and arrangements of atoms can be determined in a crystalline solid using this method. The crystallized samples, which are exposed to X-ray beams, cause the beams to diffract into many specific directions. The pattern of diffraction spots provides information about the crystal packing symmetry and size. The intensities of the spots are used to determine the

factors or parameters required to draw an electron density map. Because X-ray crystallography shows a structure of a protein–small molecule complex at very high resolution, it can clearly verify the potency of small molecules as PPI inhibitors. However, this method requires formation of a crystal for determination of the protein–small molecule complex structure and is limited in the determination of kinetics for protein–small molecule interactions that have dynamic structural properties.

4.3. Cellular validation

Cell-based assays can validate hit compounds while also revealing biological activities accompanying the biochemical changes induced by the compound binding to the target protein. This method can also offer a simple measurement of cytotoxicity. Diverse cell-based methods can be used to identify the functions and effects of the disruption of the PPI by the small molecule.

4.3.1. Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) is a method to identify physiologically meaningful PPIs using specific antibodies that indirectly capture the proteins bound to a specific target protein [87–89]. Overexpressed recombinant proteins with covalently attached tags such as Flag, Myc, and HA are frequently used to identify *in vivo* interaction between two proteins. The endogenous proteins can be also used without any modification, although this cannot be applied to proteins that are expressed at low levels, because a large amount of protein is required for detection. Potential small molecule inhibitors are administered to cells before harvest and sample preparation. If the amino acid residues of a protein that are targeted by a small molecule can be predicted through structural analysis or *in silico* docking modeling, it is also possible to verify key residues for the small molecule interaction by site-directed mutagenesis followed by analysis of the binding characteristics between the small molecule and mutant proteins [90,91].

4.3.2. Luciferase reporter assay

Luciferase reporter analysis is widely used to measure gene expression at the level of transcription [92]. Changes in gene expression attributed to modulation by the PPI can be easily measured using this reporter analysis. An expression vector containing the promoter of a gene, which responses to the PPI, fused with the luciferase gene must be prepared. For accurate measurement of the reporter gene expression, it is best to use cells with the reporter gene stably integrated into their chromosome. The role of a small molecule in the modulation of PPI can be estimated in a quantitative manner by measuring changes in luciferase activity of cells harboring a reporter gene in response to the PPI [45,93]. This method is useful for quick selection of functional compounds, with further validation possible by measurement of the transcriptional level of effector genes for the target proteins and characterization of subsequent biological activities in cells.

5. Optimization of lead compounds: synthesis of active analogs

After small molecules with potential use as drugs (i.e. leads) have been identified from above-mentioned assays, it is necessary to search for analogs to improve function and druggability. The drug properties and functionalities that can be improved include (1) structural properties such as lipophilicity, topological polar surface area, hydrogen bond acceptors and donors, molecular weight, and ionization constant; (2) physicochemical properties such as solubility and permeability; (3) biochemical properties such as metabolic stability and plasma stability; (4) safety considerations such as mutagenicity, cytotoxicity, and teratogenicity; (5) pharmacokinetics (PK) such as half-life and bioavailability. HTS and the subsequent characterization of the effective compounds result in the identification of hit compounds, but these often tend to show poor drug-like properties and hence result in failure or slowdown of drug development [94,95]. Therefore, hit compounds must be optimized through structural modifications before proceeding to clinical studies. Use of a computer aided virtual system that modulates compound–target protein interaction is an efficient and useful approach for generation of optimized compounds by chemical synthesis.

Properties such as solubility and p*K*a are taken into consideration for generation of a prodrug [96]. Solubility is one of the most important issues in the development of drugs applicable to humans. Low solubility has many unfavorable effects on *in vivo* efficacy, biological activity, and PK. Structural modifications that improve solubility include adding ionizable groups, polar groups, or hydrogen-bonding atoms, as well as reducing octanol–water partition coefficient (log *P*) or molecular weight [97]. Solubility can also be improved by formulation strategies. However, it must be carefully considered that increased solubility often results in the decreased membrane permeability of compounds. By introducing groups with differing p*K*a, both the solubility and permeability can be improved. The p*K*a can be changed by adding or removing electron-donating and -withdrawing groups, to increase the electron density at the acid or base.

The understanding of structure–property relationships (SPR) of lead compounds can provide guidelines to design synthetic analogs with diverse structures and to build preclinical models of PK–pharmacodynamics (PD) relationships [98]. Chemical synthesis to optimize efficacy of the compounds can be aided by characterization of the structure–activity relationship (SAR) in the lead compound–target protein interaction [99,100]. Thus, active analogs can be synthesized with improved efficacy, selectivity, and novelty by juggling the properties and activity of lead compounds.

6. Conclusion

PPIs had previously been considered 'undruggable,' but this is no longer true thanks to recent developments, including structural characterization of PPIs, development of screening methodologies, and evidence that the 'hot spots' regions of most PPI interfaces are compact enough to be modulated by small

molecules. These improvements have allowed a few small molecule PPI inhibitors to be successfully developed. Small molecules are more advantageous than protein/peptide drugs as therapeutic agents in many aspects, including PD and PK.

A variety of screening technologies have been developed for identification of PPI modulators by employing newly developed methods. Nevertheless, because a single screening methodology cannot rule out all false positives, validation of the small molecule–target protein interaction should be conducted through various methodologies before progressing further into the drug development process (Figure 5). Some methodologies are preferable for HTS, but others are suitable for small-scale screening based on their accuracy, cost, and time. Therefore, the development of drugs targeting PPIs requires a series of steps including confirmation of the results and modulation of the strategies. Analog synthesis using structure-based drug design is an important step for optimization of a compound's activity and druggablity.

7. Expert opinion

The pace of development of therapeutic approaches to modulate PPIs has grown over the past 20 years and led to some visible successes in the pharmaceutical industry. However, only a few PPI inhibitors have reached clinical trials and most clinical candidates did not make it through the developmental stages. This suggests that development of PPI inhibitors is intractable and should be carefully planned with consideration of therapeutic properties including efficacy, bioavailability, and toxicity.

The strategies for drug development should be considered and weighed carefully when targeting PPIs, because PPIs are clearly more challenging than other drug targets. Globular interfaces, which are formed through tertiary structure on both sides of the PPI, are too wide to be modulated by small molecules. The interfaces of PPIs that can be effectively blocked by small molecules have hot spots clustered in or around pockets containing extended binding grooves, and these small segments 'hot spots' show high-affinity in the interaction of the two proteins. As the result, small molecules can directly bind to one protein partner at the hot spots with high affinity, thus competing against the interacting partners by a mechanism known as 'orthosteric inhibition.' Meanwhile, other types of small molecules can inhibit PPIs through an allosteric mechanism in which small molecules bind to target proteins at sites distinct from the binding interface and induce conformational change of the target proteins, resulting in a hindrance of the PPI [101]. Therefore, prior to designing of PPI inhibitors, it is important to characterize the target PPI interfaces in detail and determine which types of PPI interfaces might be more manageable than others. In addition, characterization of the biological and pathological effects of the PPI is essential. Although current therapeutics targeting PPIs are mainly focused on cancer biology so far (Table 1), this approach will be valuable for the development of drugs for other diseases, because most biological and pathological processes are related to PPIs.

The Pharmacological properties of orally administrated drugs are evaluated by Lipinski's rule of 5 (RO5) [102,103]. The RO5 states that a drug should be designed to have fewer than 5 hydrogen bond donors, fewer than 10 hydrogen bond acceptors, a molecular weight below 500 Da, and a log *P* below 5. However, most PPI inhibitors are incompatible with these criteria. For example, it has been reported that among 19 PPI inhibitors identified from the literature, only eight are suitable based on RO5. Therefore, developing small molecules against PPIs outside of the rule of five is a big challenge. One strategy is synthesis of active analogs based on SPR and SAR of effective but undruggable lead compounds to improve drug-like properties such as solubility, toxicity, and the group of properties known as ADME: absorption, distribution, metabolism, and excretion. The selection of formulations or solutions applicable to humans is also important in the development of drugs. It is essential to find a balance between efficacy and druggability for successful drug development. This field has introduced many issues yet to be resolved. However, development of drugs targeting PPI is a highly valuable therapeutic approach for the treatment of diverse diseases, considering that most biological and pathological processes are mediated by PPIs. PPIs are very attractive targets in the pharmaceutical industry for the development of next-generation medicines, because they allow the pursuit of more challenging and novel targets that may represent more therapeutic value than the 'low-hanging fruit' previously pursued. Moreover, the qualitative and quantitative improvement toward understanding PPI modulation has led to further growth in drug development technologies. The advanced technologies such as new assay system for *in vitro* molecular binding, BLI, and MCT that are described in this review will be helpful in the development of more specific, effective, and safe therapeutics. Pharmaceutical industry analysts predict that worldwide sales of small molecule PPI inhibitors will reach over $800 million per year by 2018 [13]. It is possible that small molecule PPI inhibitors will occupy a large portion of the pharmaceutical marketplace in many therapeutic areas in the future.

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