Topics in Medicinal Chemistry 8

Michael D. Wendt *Editor*

Protein-Protein Interactions

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8

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Protein-Protein Interactions

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Editor
Michael D. Wendt
Dept R4N6 Bldg AP10
Cancer Research
Abbott Laboratories
100 Abbott Park Road
Abbott Park, IL 60064
USA

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Volume Editor

Michael D. Wendt

Dept R4N6 Bldg AP10
Cancer Research
Abbott Laboratories
100 Abbott Park Road
Abbott Park, IL 60064
USA

Editorial Board

Dr. Peter R. Bernstein

AstraZeneca Pharmaceuticals
1800 Concord Pike
Fairfax Research Center B313
PO Box 15437
Wilmington, DE 19850-5437
USA

Prof. Dr. Armin Buschauer

Institute of Pharmacy
University of Regensburg
Universitätsstr. 31
93053 Regensburg
Germany

Prof. Dr. Gunda J. Georg

University of Minnesota
Department of Medical Chemistry
8-101A Weaver Densford Hall
Minneapolis, MN 55455
USA

Prof. Dr. Yoshiaki Kiso

Department of Medicinal Chemistry
Director
Center for Frontier Research
in Medicinal Science
Kyoto Pharmaceutical University
Yamashina-ku
Kyoto 607-8412
Japan

John A. Lowe

jal3rd@gmail.com

Dr. Hans Ulrich Stilz

Aventis Pharma Deutschland GmbH
Geb. G 838
65926 Frankfurt a.M.
Germany

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Aims and Scope

Drug research requires interdisciplinary team-work at the interface between chemistry, biology and medicine. Therefore, the new topic-related series *Topics in Medicinal Chemistry* will cover all relevant aspects of drug research, e.g. pathobiochemistry of diseases, identification and validation of (emerging) drug targets, structural biology, drugability of targets, drug design approaches, chemogenomics, synthetic chemistry including combinatorial methods, bioorganic chemistry, natural compounds, high-throughput screening, pharmacological in vitro and in vivo investigations, drug-receptor interactions on the molecular level, structure-activity relationships, drug absorption, distribution, metabolism, elimination, toxicology and pharmacogenomics.

In general, special volumes are edited by well known guest editors.

In references *Topics in Medicinal Chemistry* is abbreviated *Top Med Chem* and is cited as a journal.

Preface to the Series

Medicinal chemistry is both science and art. The science of medicinal chemistry offers mankind one of its best hopes for improving the quality of life. The art of medicinal chemistry continues to challenge its practitioners with the need for both intuition and experience to discover new drugs. Hence sharing the experience of drug discovery is uniquely beneficial to the field of medicinal chemistry.

The series Topics in Medicinal Chemistry is designed to help both novice and experienced medicinal chemists share insights from the drug discovery process. For the novice, the introductory chapter to each volume provides background and valuable perspective on a field of medicinal chemistry not available elsewhere. Succeeding chapters then provide examples of successful drug discovery efforts that describe the most up-to-date work from this field.

The editors have chosen topics from both important therapeutic areas and from work that advances the discipline of medicinal chemistry. For example, cancer, metabolic syndrome and Alzheimer's disease are fields in which academia and industry are heavily invested to discover new drugs because of their considerable unmet medical need. The editors have therefore prioritized covering new developments in medicinal chemistry in these fields. In addition, important advances in the discipline, such as fragment-based drug design and other aspects of new lead-seeking approaches, are also planned for early volumes in this series. Each volume thus offers a unique opportunity to capture the most up-to-date perspective in an area of medicinal chemistry.

Dr. Peter R. Bernstein
Prof. Dr. Armin Buschauer
Prof. Dr. Gunda J. Georg
Prof. Dr. Yoshiaki Kiso
Dr. John Lowe
Dr. Hans Ulrich Stilz

Preface

The last decade has been a difficult one for the pharmaceutical industry. The rate of new compound registrations has slowed from its peak in the 1990s, and efforts to shorten discovery times and streamline clinical paths have not resulted in increased efficiency overall. Moreover, biologics have become an increasingly large part of the field, and their higher success rate over the last several years has further disappointed prospects for small-molecule drug discovery going forward. Several explanations for this downturn have been put forward; one of the most commonly accepted is the idea that the 'low hanging fruit' has been picked, and the drug targets remaining are simply more challenging. With the synthetic, analytical, and structural technologies present today, the far greater understanding of relevant biology, and the vast and ever-growing historical knowledge of drug discovery at our fingertips, it is difficult to imagine that current practitioners in the field of small-molecule drug discovery are simply not as proficient as those of yesterday. While regulatory issues are more challenging than ever, it seems certain that the drug targets and biological mechanisms that the industry is focusing on are simply more difficult than those of 20 or 30 years ago. Thus the identification of high-quality drug targets – targets that are not only druggable, but of high biological relevance – is more crucial than ever.

Over roughly this same period of time, better understanding of biological systems – in particular of signaling pathways and various aspects of structural biology – has brought to the fore a new type of drug target for consideration. In addition to the enzymes, ion channels, and receptors that traditionally have comprised the domain of small-molecule drug targets, protein-protein interactions (PPIs) began to gain consideration. In the last two decades, the industry has taken on many PPIs, but few compounds have gained FDA approval, the vast majority of compounds that reached clinical trials have failed in the early stages, and the overall impression is one of higher-than-average failure in the discovery stage as well. Additionally, approved compounds have for the most part been intravenously delivered, and for narrow indications, and none have achieved 'blockbuster' status. All this has served to maintain and perhaps strengthen a common view of PPIs as being undruggable. PPIs are often seen as being part of the problem, rather than part of a possible way forward.

It is certainly true that thus far, the overall effort to modulate PPIs with small molecules has not constituted a successful venture. However, comparing the last two decades of PPI-targeted drug research to the success of earlier eras, or even to more conventionally targeted work over the same period, should not be of paramount concern. The important question is whether PPIs represent viable small-molecule drug targets now, and into the future. In order to answer this, it is necessary to look at what has been learned from two decades of work on PPIs in the pharmaceutical industry, and to consider how this experience can improve prospects for PPI-targeted drug discovery in coming years.

In this volume, we look at some of the most prominent and successful campaigns within the PPI field. In an introductory chapter, the field as a whole is appraised, with short summaries of several targeted PPIs. Classification of PPIs into structural types leads to some generalizations about the small-molecule inhibitors that emerge from projects targeting them; from a drug discovery standpoint, each type also has unique positive and negative aspects. Additionally, themes of the importance of structural and mechanistic understanding of targets are highlighted.

Chapter 2 surveys the field of inhibitors of the MDM2/p53 interaction, which essentially began with the high-profile disclosure of Nutlins, and quickly and steadily grew through programs at a number of pharmaceutical companies. The MDM2/p53 interaction constitutes a paradigm example of a relatively large, hydrophobic interaction surface for which lead compounds can easily be found. The success of several programs in deriving potent, orally bioavailable small molecules attests to the druggability of this target, and compounds have begun to enter clinical trials. Additionally, the use of many of these small molecules in elucidating additional biology around the MDM2/p53 axis is described. Chapter 3 covers IAP antagonists, also referred to as Smac mimetics, which are well-represented in the clinic for the treatment of cancer. The PPI targeted here involves recognition of a short peptide sequence, and is much different in character from the MDM2/p53 interaction. Drug discovery groups consequently had very different experiences in the course of finding and optimizing chemical matter. Additionally, this chapter, like Chap. 2, illustrates the complexity of signal transduction networks, and the complementary manner in which drug discovery programs are made more difficult by this complexity of relevant biological pathways, and can be of great aid in understanding them.

Chapters 4 and 5 look at PPIs in the field of antiinfective agents, with summaries of work targeted to inhibitors of various HIV-1-related processes, and to RSV fusion inhibitors. Many of these interactions are notable for being involved in structural recognition and reorganization processes instead of signaling pathways. Therapeutic compounds modulating these interactions can operate via a number of mechanisms, from simple blockade of a necessary conformational change or of the formation of a temporary complex, to

alteration of a pre-fusion oligomeric protein into a less-functional conformation. Defining these mechanisms of action adds to the difficulty of drug design. An additional complication, deriving from mutations leading to resistant varieties of targeted proteins, further hinders discovery efforts.

Many of the most challenging PPI interfaces involve widely separated contact points, which are particularly difficult to engage with a druglike small molecule. Therefore, general methods for reproducing this family of epitopes, and thereby substituting for or augmenting the usual drug discovery process, would be of great utility. Chapter 6 presents several approaches that have been taken in the area of α-helical peptide mimetics, primarily by academic laboratories. Designed molecules of this type have already proven valuable as biological probes and have attracted attention from major pharmaceutical companies, but questions remain as to their viability as drugs. The primary obstacles to these approaches concern stability and cell permeability of the mimetics, which continue to be addressed by researchers. However, it is expected that conventional small molecules directed to this type of PPI will tend to possess particularly difficult pharmacokinetic problems of their own. In the final chapter, a small-molecule effort directed at one of these PPIs is presented. Chapter 7 is a detailed case study of the Bcl-2 family inhibitors project at Abbott. Bcl-xL and Bcl-2 have much larger binding epitopes than conventional drug targets, larger even than many other PPIs, including MDM2/p53, and this program provides a rare example of a PPI with well-separated binding pockets that has yielded a fully optimized small-molecule clinical candidate. In this chapter, the particular challenges related to this type of PPI, such as lead identification and appropriate physicochemical characteristics of inhibitors are highlighted.

I thank the authors for their contributions to this volume, and hope that in total this book presents a suitably clear picture of where the field of PPI-directed small-molecule drug discovery has been, what lessons we can take from the past, and, armed with this experience, where the field might be headed.

February 2012 Michael D. Wendt

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Contributors

Angelo Aguilar Comprehensive Cancer Center and Departments of Internal Medicine, Pharmacology and Medicinal Chemistry, University of Michigan, Ann Arbor, MI, USA

Paramjit S. Arora Department of Chemistry, New York University, New York, NY, USA

Denzil Bernard Comprehensive Cancer Center and Departments of Internal Medicine, Pharmacology and Medicinal Chemistry, University of Michigan, Ann Arbor, MI, USA

Kurt Deshayes Department of Early Discovery Biochemistry, Genentech Inc., South San Francisco, CA, USA

Stephen T. Joy Department of Chemistry, New York University, New York, NY, USA

John Kadow Department of Medicinal Chemistry, Bristol-Myers Squibb Research and Development, Wallingford, CT, USA

Sanjeev Kumar Comprehensive Cancer Center and Departments of Internal Medicine, Pharmacology and Medicinal Chemistry, University of Michigan, Ann Arbor, MI, USA

David R. Langley Department of Computer-Aided Drug Design, Bristol-Myers Squibb Research and Development, Wallingford, CT, USA

Andrew B. Mahon Department of Chemistry, New York University, New York, NY, USA

Nicholas A. Meanwell Department of Medicinal Chemistry, Bristol-Myers Squibb Research and Development, Wallingford, CT, USA

Stephen E. Miller Department of Chemistry, New York University, New York, NY, USA

Jeremy Murray Department of Structural Biology, Genentech Inc., South San Francisco, CA, USA

Richard Pracitto Department of Medicinal Chemistry, Bristol-Myers Squibb Research and Development, Wallingford, CT, USA

Domagoj Vucic Department of Early Discovery Biochemistry, Genentech Inc., South San Francisco, CA, USA

Michael A. Walker Department of Medicinal Chemistry, Bristol-Myers Squibb Research and Development, Wallingford, CT, USA

Shaomeng Wang Comprehensive Cancer Center and Departments of Internal Medicine, Pharmacology and Medicinal Chemistry, University of Michigan, Ann Arbor, MI, USA

Michael D. Wendt Dept R4N6 Bldg AP10, Cancer Research, Abbott Laboratories, Abbott Park, IL, USA

Kap-Sun Yeung Department of Medicinal Chemistry, Bristol-Myers Squibb Research and Development, Wallingford, CT, USA

Yujun Zhao Comprehensive Cancer Center and Departments of Internal Medicine, Pharmacology and Medicinal Chemistry, University of Michigan, Ann Arbor, MI, USA

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Protein-Protein Interactions as Drug Targets

Michael D. Wendt

Contents

M.D. Wendt (✉)
Dept R4N6 Bldg AP10, Cancer Research, Abbott Laboratories, 100 Abbott Park Road, Abbott Park, IL 60064, USA
e-mail: mike.d.wendt@abbott.com

Abstract Over the last two decades, a number of protein-protein interactions (PPIs) have been targeted by the pharmaceutical industry. Pharma as a whole has historically considered PPIs to be undruggable or at the very least high-risk targets, and the relative lack of success in modulating PPIs with small molecules has done little to change this prevailing view. However, many compounds are now in clinical trials, and the experiences of the last 20 years have at the very least led to improved understanding of how to approach these challenging targets. This chapter discusses some of the issues that PPIs present as targets for small molecule modulation, with emphasis on the structural characteristics of PPIs in general, and also of classes of PPIs that share specific attributes. Grouping PPIs by structural class produces a clearer picture of both the characteristics of optimized small molecules, and the relative merits and drawbacks of various PPIs as drug targets. Within this framework, much of the past work in the PPI area is summarized through capsule descriptions of efforts directed against individual targets. Some contributors to individual successes and failures, and some insights gained from the many avenues of research followed within the PPI field are put forward. Themes of the importance of understanding the structural basis of mechanism of action and of structural support for drug discovery emerge, and guidelines for future study are offered.

Keywords Drug discovery • Inhibitors • Protein-protein interactions • Protein surface

1 Introduction

It has been estimated that roughly 10% of the human genome is involved in some type of disease state, resulting in a corresponding ~3,000 potential drug targets [1]. To that one could add a number of proteins belonging to infectious organisms. However, in order to be considered suitable targets for small-molecule drug discovery, proteins also need to be "druggable" in the sense that sufficiently potent compounds can be found that also possess the physicochemical properties necessary to be orally bioavailable. Current estimates have also converged on a value of ~3,000 for the number of druggable proteins [1, 2]. The intersection of these two groups, estimated at 600–1,500 [2], defines an approximate number of exploitable small-molecule drug targets available to the pharmaceutical industry. To date, roughly 300 proteins belonging to about 130 protein families have been successfully targeted by approved drugs [3]. These numbers are dwarfed by estimates of the numbers of protein folds (1–10,000) and protein families (16–60,000) [4–6]. Historically, the vast majority of biological targets for small-molecule drug discovery have been enzymes, ion channels, and G-protein coupled and nuclear hormone receptors [1–3].

Recent advances in genomics and proteomics have brought to light vast networks of protein-protein interactions (PPIs), termed the interactome [7]. As a result, we now have a far greater understanding of signaling pathways and host-pathogen

interactions, potentially opening up a large number of new targets for pharmaceutical intervention [8–10]. However, the pharmaceutical industry has been reluctant to focus on this extremely diverse and far-reaching class of potential drug targets, at least within the framework of finding small-molecule drugs. Pharma has both recognized and successfully demonstrated the biological and commercial validity of many of these targets through the application of therapeutic antibodies, the market for which continues to lead the growth of the prescription drug market as a whole [11]. While therapeutic antibodies possess some outstanding qualities as drugs, including high stability and outstanding specificity, they also suffer from a number of serious drawbacks. Biologics are both expensive and difficult to produce, are not orally bioavailable, and are unable to antagonize intracellular protein targets. Thus, even taking into account the anticipated expansion of biologicals into additional markets, PPIs as a group remain compelling targets from a biological standpoint. Yet, while there has been a large increase in the number of small-molecule programs targeting PPIs over the last 20 years, particularly in the cancer, immunology, and antiinfective areas, a general wariness of these targets remains, stemming primarily, but by no means completely, from a negative assessment of their druggability. Moreover, the successes in this area, whether measured by the number of clinical candidates or marketed drugs, have been few in number.

This chapter will discuss what has been learned about PPIs and their viability as drug targets over the last two decades. Following a general section on the defining aspects of PPIs, highlighting the challenges that PPIs present to drug discovery groups, this chapter then looks specifically at several of the most prominent pharma-targeted PPIs over the last two decades, focusing primarily on structural characteristics of binding sites, and the issues associated with them.

2 PPIs as Drug Targets

Beyond druggability challenges, PPIs have created other complications for drug discovery programs [12–19]. These include issues of identification and characterization of small-molecule binding sites, the lack of natural ligands that can be easily used as a starting point for drug discovery, and various problems associated with compound screening – relying on screening sets that are poor matches for PPIs, separating real from artifactual hits, and verifying the proper binding location of hit compounds. All of these issues ultimately relate back to the fundamentally different endogenous interactions of PPIs, compared to enzyme-substrate or receptor-substrate interactions. Close examination of this class of interactions follows.

2.1 Structural Characteristics of Protein-Protein Interfaces

While in principle binding between proteins can be driven by widely varying mixtures of polar and electrostatic interactions (H-bonds and salt bridges),

in practice the binding energies are largely derived from the mutual burying of largely complementary hydrophobic surfaces [20]. The interfaces formed normally have a hydrophobic core, with a more polar border which remains solvent-accessible after binding [21]. These interface cores are often the most hydrophobic patches on the individual protein surfaces [22]. Both theory and observation have established that a rough minimum of 1,200 $Å^2$ of interface area, or 600 $Å^2$ per monomer, is required to stabilize a PPI [20]. Most PPIs of interest will involve somewhat larger surface areas, between 750 and 1,500 $Å^2$ for each protein [23]. It has been suggested that the minimum area corresponds to that required to make a water-tight seal around a set of interactions that are strong enough to carry out biological activity [21].

It is important to make a distinction between obligate, permanent dimer or oligomer interfaces forming a stable structure, and transient interfaces formed by separate proteins capable of carrying out independent functions. Members of the former group bind much more strongly, and their interfaces are larger and less flat, with tighter packing and very few water molecules trapped between monomers [20, 23–25]. Also, the energetic dominance of hydrophobic surface matching for obligate protein-protein complexes is more overt than for temporary complexes [23, 26, 27]. Accordingly, the amino acid compositions of obligate interaction surfaces are much more hydrophobic than overall protein exteriors, sometimes closely resembling those of protein cores [26–30], while transient complex surfaces are roughly halfway between interior and surface by this measure [23, 24]. This is to be expected given the amounts of time the two types of surface spend exposed to solvent.

Relative to solvent-accessible surfaces, permanent interfaces contain an over-abundance of nonpolar amino acids, both aromatic and aliphatic, particularly near the center of the contact surface. Temporary interfaces do not feature a much higher percentage of nonpolar residues, but do contain more neutral polar residues at the expense of charged amino acids, with the exception of arginine, which is also overrepresented in obligate complex surfaces [21, 23, 24]. Hydrogen bonds are not uncommon in temporary interfaces, with, on average, one hydrogen bond per 170 $Å^2$ of interacting surface [23]. About one third involve a charged side chain, and salt bridges comprise just over one in ten polar interactions. Among obligate complexes, the density of hydrogen bonds is not very different [20], but a far greater percentage involve one or two charged species, in keeping with relative surface amino acid compositions [30].

Among nonpolar residues, the larger amino acids, and in particular the aromatic residues, are overrepresented in the hydrophobic cores of transient PPI surfaces [21, 23, 31–33]. While still lipophilic, aromatic side chains have lower transfer free energies per atom than aliphatic side chains [34–36]. Methionine, while not a major component of interfaces, is also often more frequently found there than in permanently exposed or buried surfaces, and also has a low transfer free energy. Methionine is known to interact very favorably with aromatic groups, probably due to the polarizability of both aromatic systems and sulfur atoms. Together, these residues also feature the most flexibility and size among nonpolar residues, and thereby have

the greatest capacity to change the shape of a local surface; their overrepresentation enhances the well-known mobility of many hydrophobic patches.

To the medicinal chemist, then, a first-order description of protein-protein interfaces paints a picture of two very large interacting surfaces, mostly devoid of small, polar features that could by themselves, bound to an appropriate structure, produce large amounts of affinity. The surfaces instead achieve sufficient affinity only through summing weak interactions over a very large area. Optimization of antagonists of this type of interaction are expected to yield very large, hydrophobic, and atom-inefficient compounds.

An important refinement to this view was put forth by Clackson and Wells [37], who demonstrated, through the use of alanine scanning [38], that certain key residues in a growth hormone system had a very disproportionate effect on binding, and the corresponding subregions of binding surfaces were referred to as "hot spots." Similarly, multiple studies have established that a very limited number of residues at protein-protein interfaces are crucial, and a single point mutation can often greatly reduce affinity or even completely block a PPI without changing the overall integrity of the protein [39–41]. Hot spots have since become the subject of a great deal of study, particularly through their interactions with small-molecule inhibitors of PPIs [21, 23, 24, 42–46]. Hot spots tend to be of small-molecule size and are normally located near the center of PPI surfaces. Hot spots also commonly have a capacity to adapt conformationally, producing more concave local topologies and increasing their ability to accommodate more conventionally drug-sized ligands. Many studies using phage display of small peptides have shown strong preferences for binding at hot spots [47–49], which illustrates their propensity for binding a variety of small molecules. Finally, a number of publications have demonstrated the robustness of the hot spot paradigm by using hot spot-based models to rationally predict small-molecule binding sites on proteins [42, 50–54].

While the conformational flexibility of hot spots is not expected to greatly reduce the lipophilicity of a corresponding small-molecule inhibitor, it does improve the prospects for a viable antagonist of a reasonable size. By forming a better defined pocket, a hot spot may allow more protein surface to be buried by a smaller ligand. Still, due to the relative lack of high-energy polar interactions between protein and ligand, efficiencies of binding [55, 56] of optimized PPI-directed compounds will still be lower than those of most receptor- or enzyme-targeted compounds. However, the differences, while meaningful, are not so extreme. In one study, a group of optimized PPI inhibitors had an average ligand efficiency (LE) of 0.24, compared to 0.25–0.35 for protease inhibitors, and 0.3–0.4 for kinase inhibitors [16]. A more recently derived figure gives a value of 0.27 for advanced PPI inhibitors [57]. Thus, while the view that PPI binding is primarily driven by hydrophobic surface-matching is largely correct, the impact of this on the druggability of many PPI sites is commonly lessened due to the presence of small subsites within the contact surfaces.

2.2 Physical Characteristics of Small-Molecule PPI Antagonists

As alluded to above, the characterization of PPIs as undruggable rests on a view of their binding surfaces as being in general too large and too hydrophobic for their constituent interactions to be inhibited by druglike molecules. Rules of thumb such as Lipinski's Rule of Five [58], polar surface area (PSA) restriction [59], and the "golden triangle" [60] seek to quantify measurable aspects of orally bioavailable molecules in order to provide guidance to future medicinal chemistry efforts. Compounds in violation of these measures of druglikeness are expected to be particularly prone to preclinical and clinical failures associated with various ADMET issues.

As the amount of work targeting PPIs has grown, many researchers have analyzed data from the literature, from both academic and industrial research groups, to derive chemotypes for PPI inhibitors [57, 61–63]. Many different interactions, involving most protein fold classes, have been included in these efforts. As a result, a general picture is emerging of the molecular properties of advanced PPI inhibitors. These compounds, as expected, usually fall outside the standard realm of druggable compound space, having higher molecular weight and logP values than rules of thumb recommend. Even early hits and leads often suffer by comparison to similar guidelines established for these compounds [64–66]. This is a particular problem because early project molecules tend to become more lipophilic throughout the optimization process [64]. Interestingly, advanced PPI inhibitors also tend to be relatively rigid and are more highly aromatic than other optimized compounds, also in keeping with what is known about the makeup of hot spots [19, 57, 61, 62].

The failure of optimized PPI antagonists to fall within desirable ranges of compound space has reinforced the concerns of pharmaceutical discovery programs over the druggability of PPIs. Yet implicit in these assessments are a number of assumptions. First, the common physicochemical guidelines defining "druglike" molecules may be too restrictive, leading to a similarly restrictive view of how many druggable proteins exist. Second, the definition of some potential targets as undruggable due to either a lack of precedent, or previous failures to find compounds from screening efforts, lacks sufficient rigor, and is dependent on an assumption of the future of drug discovery looking very similar to the past.

Good aqueous solubility and high permeability are optimal qualities for a compound to have in order to possess good oral bioavailability. In practice, candidate molecules, particularly larger compounds, must try to strike a balance between these two characteristics. It comes as no surprise that PPI inhibitors usually fail on these grounds. If limits on polar surface area (PSA) and logP are adhered to, then molecules above a certain size are mathematically almost certain to run afoul of one of those two guidelines. A noted study by Veber [67] demonstrated that low PSA and low rotatable bond count are by themselves good predictors of oral bioavailability, suggesting that molecular weight is primarily a proxy for other characteristics, especially molecular flexibility. Additionally, several papers have

shown that oral absorption can be predicted with a high degree of accuracy from surface properties such as PSA and the number of hydrogen bonding groups alone [59, 68–70]. Interestingly, these correlates roughly describe the set of advanced PPI inhibitors discussed above, which are relatively rigid and have low PSA values and few hydrogen bonding groups in spite of their size. It may be that by keeping these parameters low, higher than recommended logP values and molecular weights may be tolerated. Finally, PPI inhibitors, by virtue of their larger size, will in general be expected to have a greater tendency to reduce their effective size and polarity through hydrophobic collapse or internal hydrogen bonding [71], further enhancing their membrane permeability.

Oral bioavailability rules are based on successfully developed compounds discovered years or decades ago. However, current and recent medicinal chemistry work has not been targeting the same therapeutic modalities as in past decades, and compounds designed toward current targets often do not and cannot occupy the same chemical space as older marketed drugs. Several studies over the past decade have demonstrated that advanced compounds have become larger and more hydrophobic as the industry has moved on to different targets [72–75]. Additionally, compounds directed to individual targets have also tended to increase in size over time [76, 77]. A number of explanations for this phenomenon have been put forward [75], among them the dominance of the target-based drug discovery paradigm, an increasing focus on selectivity, the need to find new patentable space, and reliance on high-throughput screening (HTS) for chemical matter.

The foregoing is not meant to suggest that concerns over the likely physical properties of PPI inhibitors should be ignored. Several studies have shown that clinical attrition rates of compounds correlate with size and lipophilicity [72, 74, 78], and the trend within pharma toward deriving compounds with that profile has certainly contributed to difficulties in getting clinical candidates to market. It is only meant to point out that comparison of current targets of any type to those responsible for populating recommended chemical space places an – unfortunately – unrealistic burden on them. A more appropriate and important comparison is between PPIs and other current potential drug targets. Targets such as HIV protease, and peptide-liganded GPCRs such as the angiotensin II and chemokine receptors, have become increasingly popular, and inhibitors directed to these targets are often near or beyond desired ranges for prediction of good oral bioavailability. Many kinase inhibitors, particularly inactive-conformation-targeting compounds, also share these traits. These high molecular weight compounds often have very low aqueous solubility but fair to good lipid solubility and adequate oral pharmacokinetics [79]. Against this background, the higher-than-historical size and lipophilicity of PPI-modulating compounds, while far from optimal and of legitimate concern, need not be seen as an overriding obstacle, but as one complicating factor – among many that are inevitably presented by a given drug target – common to PPIs, as well as several non-PPI targets currently under examination by the pharmaceutical industry.

Compounds with these physicochemical characteristics will normally fit into the FDA Class II or IV pharmacokinetic classifications. Many may be expected to exhibit dissolution rate-limited absorption, due to their low solubility and high lipophilicity. One analysis has shown that poorly soluble drugs that have nevertheless reached the market tend to have low solubility driven by lipophilicity, and not high crystallinity, and this type of compound is also most susceptible to enhancement of absorption by the implementation of various excipient additions or advanced formulation techniques [80]. A number of formulation technologies have been introduced in recent years that address pharmacokinetic liabilities such as this, among them protein nanoparticles [81], gels [82], microemulsions [83], dendrimers [84], liposomal nanocarriers [85], and melt extrusions and other solid dispersion technologies [86]. Increasing numbers of compounds entering the clinic, not only PPI inhibitors, will inhabit a different region of chemical space than older compounds, and enabling technologies will increasingly be of value in enhancing their oral bioavailability. Further experience will be necessary to determine whether poorly soluble, high logP compounds can frequently enough achieve acceptable oral bioavailability through maintenance of PSA, and application of formulation technologies.

2.3 Identification of Small-Molecule Binding Sites

Identifying chemical matter for a PPI can be particularly challenging. An early difficulty associated with some PPI drug targets is the identification of the proper location of intervention by a small molecule. Unlike traditional drug targets, for which the natural ligand is of small-molecule size and the location of binding is known, the small-molecule binding site of a PPI – if it exists at all – must be determined. Usually, the endogenous substrate is of such a size that there is still a question as to where within that interaction surface a small molecule might bind.

The average volume of concave pockets at PPI interfaces is very small, with an average of six sites of roughly 50 $Å^3$ per interface [54]. It is extremely difficult to identify from simple inspection which of these are hot spots exploitable by small molecules. This is particularly evident from comparisons of unbound and complexed structures of a number of proteins involved in PPIs [46, 87]. A given protein surface should be considered to be able to assume a number of different local conformations, with only some of them featuring well-defined, deep binding pockets capable of a strong interaction with a given ligand. An excellent illustration of this is a study using picosecond time-scale molecular dynamics simulations on MDM2, IL-2, and Bcl-xL that showed the unbound proteins forming transient conformations similar to their inhibitor-bound states [88]. Thus, it is usually not clear from inspection of a given apoprotein or endogenous protein-ligand crystal structure where hot spots are, nor what their local conformations would be given the presentation of an appropriate small molecule [89, 90]. Methods to determine hot spot locations include mutagenesis studies, including the aforementioned phage display

methodology [47–49]. Alanine scanning in particular has been useful in determining which parts of a peptide epitope are responsible for key contacts [45, 47]. Additionally, both molecular dynamics studies and computational methods based on small-molecule docking or surface scoring functions [42, 50–54, 91], can aid in predicting hot spots, but these latter methods by themselves may not provide the assurance of druggability necessary to begin a drug discovery program.

2.4 Compound Screening Methods

The PPI inhibitor discovery process often begins with attempts at dissection of the 'lock' member of the binding pair into a minimal binding peptide. Some PPIs center on a short peptide binding motif; for these, epitope mapping of this sort is often successful. In most cases, however, this approach is not possible, as there is no single dominant part of the binding surface that can be located to a continuous peptide sequence. The key contacting amino acid residues – hot spots – are widely separated, both spatially and in terms of secondary structure, and the impracticality of using an α-helical or β-sheet fold as a starting point for small-molecule drug discovery means that other avenues must be sought. For these reasons, HTS is often applied to the problem.

Many types of assay are available to be used in HTS protocols to identify inhibitors of PPIs, but a competition assay, in which inhibition of complex formation is measured, is most common. Fluorescence polarization (FPA), fluorescence resonance energy transfer (FRET), enzyme-linked immunosorbent assays (ELISA), and other assay formats have been used. The interacting proteins can be used in their full-length forms; though, more frequently only the interacting domains are employed, and if possible the excised interacting peptide is usually preferred.

PPIs often confront HTS with a number of complications. First, the matter of separating true hits from artifacts, compounds that cause precipitation or aggregation of the protein, can be particularly difficult. When screening against a hydrophobic PPI, there is virtually no chance of finding very high affinity (sub-micromolar) compounds, so the best hits will be at or below an affinity level where artifact compounds are more common. Moreover, for well-established target types, screening will normally yield a group of compounds among which some contain a functionality that is known to, or can easily be identified to, interact strongly with a key moiety within the target. Compounds without such a moiety can often be excluded with a high degree of confidence. For PPI targets, compounds that are aggregators [92, 93] or denaturants – acting via a detergent effect – may not be separable from true hits by inspection, especially in light of the fact that both types of promiscuous hits tend to be populated by large hydrophobic compounds. Careful modification of assay conditions can often diagnose aggregation; alternately, analytical ultracentrifugation (AUC) can be used [94–96]. Additionally, AUC, isothermal titration calorimetry (ITC) [97, 98], surface plasmon resonance (SPR) [99, 100], and other methods can determine binding stoichiometry. Also, questions