SCRIP Insights

Advances in the Discovery of Protein-Protein Interaction Modulators

Key strategies, biological targets, technologies, successes, and remaining challenges

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Executive summary

Introduction

- The pharmaceutical industry is in crisis, due to falling revenues caused by the loss of patent protection of major blockbuster drugs, and the failure of R&D to produce new products that could make up for or exceed the lost revenues. Companies are responding by cutting their budgets (including R&D budgets), and by attempting to outsource more and more R&D functions, including discovery research.
- However, dependence on outsourcing of discovery research for large portions of pharmaceutical pipelines remains unproven, and simply cutting R&D investment is highly likely to decrease R&D output. To ensure companies' growth and survival, it will be necessary to directly fix the issues that have been attenuating R&D productivity.
- Recent analyses single out target selection as the key factor that is limiting the productivity of pharmaceutical R&D in the current era. The conventional wisdom is that researchers are running out of druggable targets that could be used for the discovery of novel drugs. However, as of 2011, 75% of protein research has focused on the 10% of proteins that were known before the mapping of the human genome. This is despite the finding that many proteins that were identified during the mapping of the human genome are linked to disease.
- In addition to novel druggable targets, there are emerging more challenging targets that are especially relevant to disease. Chief among these are protein-protein interactions (PPIs). Medicinal chemists have previously considered PPIs to be undruggable. However, there has been recent progress in developing small-molecule and peptide drugs designed to address PPIs. Since the human interactome includes between 130,000 and 650,000 PPIs, even if only a small fraction were relevant to disease and could be addressed by drugs, PPIs would still constitute an enormous are of unaddressed target opportunity.
- Current concepts of druggability are centered on the use of combinatorial chemistry and high-throughput screening in drug discovery, coupled with the Lipinski "Rule of Five," which predicts the "drug-like" properties of a small organic compound. This dominant paradigm has, for example, greatly diminished the role of natural products in drug discovery. The poor coverage of chemical space by

combinatorial libraries has limited researchers' ability to discover drugs for challenging targets, especially PPIs.

- The contact surfaces involved in PPIs are large and lack the types of cavities present in the surfaces of proteins that bind to small-molecule ligands. PPIs also do not have natural small-molecule ligands that can be used as starting points for drug design. Despite these challenges, focusing on "hotspots" in PPI interfaces can enable researchers to design small-molecule PPI modulatory drugs.
- There are marketed natural product drugs that modulate PPIs, such as the vinca alkaloids, colchicine, cyclosporine, and tacrolimus. Moreover, several more-recently discovered PPI inhibitors are in clinical development, and one, the thrombopoeitin mimetic eltrombopag (Ligand/GlaxoSmithKline's Promacta/Revolade), has reached the market. This indicates that it is feasible to discover and develop small-molecule PPI modulators.

General strategies for targeting protein-protein interactions with small molecules

- Researchers have developed a set of tools for discovering small-molecule drugs that target PPIs, which have been used to discover all the compounds that have so far reached the clinic. These include alanine scanning mutagenesis, X-ray crystallography, and such fragment-based drug design (FBDD) methods as tethering and structure-activity relationship by nuclear magnetic resonance (SAR by NMR). These have been combined with such methods as screening of chemical and natural-product libraries using cell-based assays.
- This set of technologies has not been powerful enough to enable the regular and consistent discovery of small-molecule PPI modulators. Compounds have so far been discovered on a "one compound at a time" basis.
- However, in recent years researchers have developed new technologies that have the potential to move this field up the technology development curve and enable the regular discovery of small-molecule PPI modulators.

- A key innovation is computational solvent (CS) mapping, which enables the virtual identification of druggable binding sites within PPIs, and the subsequent discovery or design of small-molecule PPI modulators.
- Another important advance has been in the design of improved chemical libraries for targeting PPIs, via such methods as diversity-oriented synthesis (DOS) and the synthesis of macrocyclic compounds via Ensemble Therapeutics' DNA-programmed chemistry (DPC) technology.
- These new technologies are combined with cellular screening assays (including high-content screening) and medicinal chemistry to discover or design PPI modulators.
- Ensemble Therapeutics and Forma Therapeutics are particularly prominent in applying PPI-focused technologies. Both have discovered preclinical-stage small-molecule PPI inhibitors, and both have attracted alliances with big pharma companies, specifically Novartis, Pfizer, and Bristol-Myers Squibb.
- Companies had previously been abandoning or cutting back on their PPI modulator discovery efforts. However, as a result of the development of the new suite of enabling technologies, both venture capital investment and companies – including big pharma – have been moving back into PPI discovery. Several other companies are continuing to develop compounds that resulted from earlier now discontinued or reduced PPI modulator discovery programs (e.g. GlaxoSmithKline (in collaboration with Ligand), Roche, and Abbott).
- With the new strategic importance of PPIs as targets, we expect additional companies to enter the field.

Small molecules targeting protein-protein interactions of cell-surface receptors

- Many cell-surface receptors (e.g. receptors for cytokines, chemokines, and growth factors, as well as integrins) exert their physiological functions by interacting with proteins or peptides.
- Researchers and companies have developed large-molecule drugs – including recombinant proteins and mAbs – that target many of these receptors. These include biologic inhibitors of tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF), and recombinant versions of proteins that are ligands for cell-surface receptors, such as epoetin alfa (Amgen's Epogen / Johnson & Johnson's

Procrit), and granulocyte colony-stimulating factor (G-CSF) (Amgen's filgrastim/Neupogen.) These biologics are among the most successful drugs on the market today and, in the case of agents whose patents have expired, they are the targets of biosimilar development.

- Some researchers and companies would like to develop small-molecule receptor modulators to substitute for these biologics. Such small-molecule drugs might be orally available, and would have the potential to be less expensive and safer than the corresponding biologics. In some cases, developing biologic drugs to modulate such receptors has not been possible, so any small-molecule receptor modulators would be the first drugs to be developed that address these receptors.
- The development of small-molecule drugs that modulate these receptors has been difficult. Nevertheless, three such drugs have reached the market: eltrombopag (Ligand/GSK's Promacta/Revolade), maraviroc (Pfizer's Selzentry/Celsentri), and plerixafor (Genzyme's Mozobil).
- Other compounds are in development, including Ligand/GSK's GSK2285921/LGD-4665 (a follow-up compound to eltrombopag; Phase II), Ligand's early-stage EPO and G-CSF agonists, SARcode's ophthalmologic compound SAR1118 (Phase III), Ensemble's macrocyclic TNF antagonists, and numerous chemokine receptor antagonists, including ChemoCentrix/GSK's Phase III Crohn's disease agent Traficet-EN/GSK786.

Small molecules targeting intracellular signaling pathways

- Signal transduction and intracellular signaling pathways are fundamental to cell and organism physiology, in health and disease. They often become dysregulated in cancer, metabolic diseases, immune diseases, and other major diseases.
- Although drug developers have successfully targeted tractable signaling receptors with small-molecule drugs and with biologics for many years, intracellular signaling pathways have until relatively recently been inaccessible to drug discovery researchers. The discovery and development of kinase inhibitors, which began in the late 1990s/early 2000s, represents a very significant breakthrough.
- Many intracellular signal transduction pathways that are dysregulated in cancer and other diseases remain inaccessible to drug discovery researchers, since they contain few if any "druggable" targets,

and are driven by key components that have so far been intractable. PPIs are critical components of all signaling pathways, and researchers would like to find ways to address the “undruggable” PPIs that are central to these pathways.

- Academic researchers at Harvard Medical School, in collaboration with Novartis, have discovered several small-molecule inhibitors of the Tcf/β-catenin PPI, a transcription factor complex that is central to the Wnt pathway. This pathway is dysregulated in subsets of several types of cancer, especially colorectal cancer, multiple myeloma (MM), hepatocellular carcinoma (HCC), and B-cell chronic lymphocytic leukemia (B-CLL). This research involved structural and mutagenesis studies of the PPI (which identified a hot spot), followed by assay development and screening of natural product libraries. Novartis researchers have concluded that the compounds identified warrant further studies to determine the feasibility of testing them in human clinical trials.

- Academic researchers led by Ari Melnick (Cornell University Medical College) have discovered a small-molecule inhibitor of the BCL6/SMRT PPI in diffuse large B-cell lymphoma (DLBCL). The researchers performed structural and mutagenesis studies (which identified a hot spot), performed computer-aided drug design, and used their models for virtual screening of 1,000,000 commercially available compounds. Compound selection was based on chemical diversity, drug-likeness, immediate commercial availability, and ability to block BCL6-mediated transcriptional repression in a cellular assay. The researchers identified a lead compound, which specifically killed BCL6-positive lymphoma cell lines and BCL6-positive tumor cells in xenograft models. The researchers are optimizing their lead compound to develop a clinical candidate for BCL6 targeted therapy for DLBCL.

- Researchers at the Leibniz Institute for Molecular Pharmacology have discovered small-molecule A-kinase anchoring protein (AKAP)-protein kinase A interaction disruptors for the potential treatment of chronic heart failure. AKAPs are scaffolding proteins that tether protein kinase A (PKA) and other signaling proteins to specific intracellular sites. The tethering of PKA (also known as cAMP-dependent protein kinase) via a PPI with an AKAP results in the compartmentalization of cAMP signaling within the cell. The AKAP18δ isoform serves as a scaffold for organizing the adrenaline-beta-adrenoreceptor-cAMP-PKA signaling pathway in cardiac muscle cells. The researchers developed a screening assay for disruption of the AKAP18δ-PKA PPI, and screened a library of over 20,000 “drug-like” compounds.

They identified nine compounds, and selected one of them, FMP-API-1, for further studies. FMP-API-1 disrupted the AKAP18δ-PKA PPI with a micromolar dissociation constant, and worked via an allosteric mechanism. Higher affinity drug-like small-molecule AKAP-PKA PPI antagonists are still sought.

- As illustrated by the three case studies in this chapter, researchers have shown that it is possible to target PPIs involved in intracellular signaling pathways with small-molecule agents, producing “tool compounds” that enable these signaling pathways to be probed. In some cases it has been possible to achieve sufficient optimization to identify compounds that can be taken into the clinic.

Small molecules targeting the ubiquitin system

- The ubiquitin system is a fundamental regulatory system in all eukaryotic cells, comparable in importance to protein phosphorylation. This system is best known for regulating intracellular protein turnover but is also involved in such functions as regulation of mitosis and innate immunity, and regulation of certain protein kinases and other enzymes.
- The ubiquitin system is based on covalently linking the small (8.5kDa) regulatory protein ubiquitin – either singly or as polyubiquitin chains – to numerous specific protein targets. In protein degradation the ubiquitin system works together with the proteasome in a pathway known as the ubiquitin proteasome system (UPS). In the UPS, ubiquitin is used to tag proteins for degradation by the proteasome.
- Many researchers see the ubiquitin system as a virtually untapped area of opportunity for drug discovery and development, potentially comparable in importance to protein and lipid kinases. The first approved drug, the proteasome inhibitor bortezomib (Millennium’s Velcade), was approved in 2003.
- The ubiquitinylation pathway is complex and involves several levels of mediators, principally E1 ubiquitin activators, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. In this pathway, ubiquitin moves from E1s to E2s, and E3s interact via PPIs with ubiquitinylated E2s and with substrate proteins. In these complexes, ubiquitin is transferred from the E2 to the substrate. This process may be repeated, resulting in tagging of substrates with polyubiquitin chains.

- As one moves down the ubiquitinylation cascade, the degree of specificity of the process, and therefore the potential for development of specific drugs, increases. There are only 10 E1s encoded in the human genome, but there are about 40 E2s and over 600 E3s.
- Drugs in clinical development that target the UPS include second-generation proteasome inhibitors, and two agents that target the ubiquitinylation pathway itself. These include Millennium's MLN4924, which inhibits a pathway that activates one class of E3s, the Cullin RING E3 ligases (CRLs), and Celgene's CC0651, an allosteric modulator that inhibits the ubiquitinylation activity of an E2 that interacts with CRLs. These agents, which are both enzyme inhibitors, appear to be more specific than proteasome inhibitors, but their targets ubiquitinylate hundreds of substrate proteins.
- The most specific agents that target the ubiquitin system would be agents that target E3s. Since E3s interact with their substrates via PPIs, such agents would be PPI modulators. Thus the intractability of PPIs has been a major bottleneck to the development of specific agents that target the ubiquitin system.
- Researchers have developed agents that target one E3. This is HDM2, which is the human homolog of mouse double minute 2 (MDM2) protein. HDM2 interacts with p53 via a PPI. p53, the "guardian of the genome," controls pathways that respond to DNA damage or other insults by blocking cell proliferation, and then either inducing DNA repair or inducing apoptosis.
- p53 is mutated or inactivated in nearly all human cancers, which allows these cancers to proliferate continuously and renders them resistant to cytotoxic chemotherapy. In approximately 50% of human cancers, p53 is inactivated via mutation. In the other 50%, p53 remains unmutated, but is inactivated. The main means of inactivation is via HDM2, which is overexpressed in the majority of cancers with wild type p53.
- HDM2 regulates p53 in three ways: inhibition of p53-induced transcription, promotion of export of p53 out of the nucleus, and inducing p53 degradation by the proteasome. Both of the latter activities involve HDM2's E3 ubiquitin ligase activity.
- There are currently two leading drug candidates that specifically disrupt the HDM2/p53 PPI. The most advanced compound, now in Phase I clinical trials, is Roche's RG7112, which is an analog of nutlin-3a.

The other compound is Ascenta/Sanofi's MI-219, analogs of which are currently in advanced preclinical studies.

- Johnson & Johnson's JNJ-26854165, which is in Phase I clinical trials in advanced or refractory solid tumors, was an agent with an unknown mechanism of action that had been deemed to target the HDM2/p53 PPI. However, recent studies indicate that it works via a different mechanism of action that does not involve HDM2.
- Given the large number and specificity of E3 ubiquitin ligases and their important role in intracellular pathways, there is a large field of possibility for discovery of novel PPI modulators that target these biomolecules and their interactions with their substrates.

Small molecules targeting protein-protein interactions that control apoptosis

- Apoptosis is the major and best-studied pathway of programmed cell death in all multicellular animals. It is an ATP-dependent, programmed, and orderly process of cellular suicide. Apoptosis is essential for normal embryonic development, and for maintaining normal cellular homeostasis in adults, as well as for response to infectious agents. It is dysregulated in several major diseases. Cancer is the major focus of researchers seeking to develop drugs that modulate apoptotic pathways, since apoptosis is blocked in perhaps all cancers. This is a significant factor in uncontrolled cellular proliferation in cancer.
- The central pathways of apoptosis are controlled by a complex system of pro- and antiapoptotic Bcl-2 family members, which act to ensure that apoptosis is only triggered when it is appropriate. Bcl-2 family member interactions that control apoptosis are PPIs. Thus it has been difficult to discover agents that affect the central pathways of apoptosis and which are capable of being taken into the clinic.
- There are now two Bcl-2 family PPI disrupting agents in clinical trials: Abbott/Genentech's navitoclax and Gemin X/Cephalon/Teva's obatoclax.
- Navitoclax (ABT-263) is the result of the fragment-based drug discovery methodology known as SAR by NMR, which led to a Bcl-2 inhibitor designated as ABT-737. The researchers later optimized

ABT-737 to produce a second-generation compound, navitoclax, which has improved physicochemical and pharmacological properties and is orally available.

- Abbott and Genentech are codeveloping navitoclax. It is in Phase I and Phase II clinical trials in various cancers, including as a combination with targeted therapies such as Rituxan (rituximab) and Tarceva (erlotinib), as well as with cytotoxic chemotherapies and as a single agent.
- Obatoclax (GX15-070) was discovered by Gemin X, which in 2011 was acquired by Cephalon; later in 2011, Cephalon was acquired by Teva. The Gemin X researchers discovered the drug by screening natural product libraries, and optimizing the resulting lead compound.
- Obatoclax is a potent inhibitor of Mcl-1. Mcl-1 is overexpressed in several types of cancer and undergoes rapid steady-state turnover by the 26S proteasome complex. As a result, treatment of malignancies with proteasome inhibitors like bortezomib results in accumulation of Mcl-1. This can result in interference with the therapeutic response to bortezomib. This suggests that a good Mcl-1 inhibitor such as obatoclax might be useful in these cases in which Mcl-1 is important in blocking apoptosis.

Stapled peptides for targeting protein-protein interactions

- In parallel with the discovery and development of small-molecule PPI modulators, and of second-generation technologies for the discovery of such compounds, other researchers have been designing peptides that mimic protein domains that are involved in PPIs. This involves mimicking not only the amino acid sequence of these domains, but also their secondary structure. It has been proposed that “stapled peptides,” which contain a small loop rigidifying the peptide conformation, will able to penetrate cells, be resistant to degradation by proteolytic enzymes, and have favorable pharmacological properties.
- The private, venture capital-funded discovery-stage biotechnology company Aileron Therapeutics (Cambridge, MA) was founded in 2005 to develop and commercialize the stapled peptide technology invented by Gregory Verdine (Harvard University) and his colleagues

- Aileron has been building a pipeline of stapled-peptide agents targeting Bcl-2 family members that control apoptosis, the HDM2/p53 PPI (for p53 reactivation), and the notch signal transduction pathway. It also has an HIV1 capsid formation inhibitor (a potential anti-HIV/AIDS drug).
- Roche has entered into a collaboration with Aileron to discover, develop and commercialize stapled peptide agents against undisclosed targets in cancer, virology, inflammation, metabolism and neurology. Aileron announced in November 2011 that it had achieved a key milestone related to *in vivo* proof of concept in one of the programs in which it is collaborating with Roche, thus triggering a milestone payment. The Roche/Aileron agreement constitutes an important validation of Aileron's stapled peptide technology and pipeline.
- Stapled-peptide technology for the development of PPI modulators represents a promising alternative to the development of small-molecule PPI modulators. However, stapled peptide agents are – unlike numerous small-molecule PPI modulators – not orally available. Moreover, as yet not one of Aileron's agents has entered the clinic. Thus the stapled peptide field awaits the achievement of proof of concept in human studies.

Outlook for protein-protein interaction modulators

- The discovery and development of protein-protein interaction modulators has been difficult, due to the structure of protein-interacting interfaces, the lack of natural ligands for PPIs to serve as starting points for drug design, and the unsuitability of proprietary pharmaceutical company or commercially available chemical libraries for use in HTS campaigns to identify compounds that modulate PPIs.
- Despite the difficulty of discovering and developing PPI modulatory drugs, developing PPI modulators is becoming of increasing strategic importance to the pharmaceutical and biotechnology industry. Companies need to address hitherto "undruggable" targets, especially PPIs, to reverse the low productivity of pharmaceutical R&D. It is estimated that all known small-molecule drugs address only 2% of human proteins. Most of the remaining proteins are designated "intractable." Most of the proteins that are critically involved in disease pathways lie within the category of intractable targets, and most of these targets are PPIs.

- Despite the difficulties in discovering small-molecule PPI modulators that are capable of being taken into clinical trials, researchers and companies have done so in several cases. Central to these successes has been the determination of “hotspots” in protein-protein interfaces. By targeting hotspots, several compounds that directly modulate PPIs have been discovered. However, this has been on a sporadic “one compound at a time” basis, which has usually been slow and laborious.
- Among these examples is one compound that has reached the market – eltrombopag (Ligand/GSK’s Promacta/Revolade) – as well as several others currently in clinical trials. In addition to these direct PPI modulators, there are also allosteric chemokine receptor modulators, including two marketed drugs – maraviroc (Pfizer’s Selzentry/Celsentri) and plerixafor (Genzyme’s Mozobil) – and several others now in clinical trials.
- The sporadic, “one compound at a time” approach cannot meet the strategic needs of the pharmaceutical/biotechnology industry to expand the numbers of targets that can be addressed by developable drugs. Because of the difficulty in making PPI modulator development a commercial proposition on a consistent basis, all the pioneering companies in this field (e.g. Genentech, Roche, Sunesis, and Abbott) abandoned or cut back on their PPI modulator programs, and several of the pioneering industry researchers in the field have moved to academic positions.
- Researchers have been developing second-generation technologies designed to enable the development of small-molecule and peptide PPI modulators on a more consistent basis. These include computational solvent (CS) mapping, diversity-oriented synthesis of chemical libraries, Ensemble Therapeutics’ proprietary DNA-programmed chemistry (DPC) technology for synthesis of libraries of macrocyclic compounds, and stapled-peptide technology.
- Responding to these new technologies, pharmaceutical and biotechnology companies are moving back into the PPI modulator field. In addition to companies developing compounds discovered via older technologies that are already in clinical trials, companies active in PPI modulator discovery include Ensemble, Forma, Aileron, Bristol-Myers Squibb, Pfizer, Novartis, Ajinomoto, Ligand, Ascenta, Sanofi, Cephalon/Teva, and Roche. In several cases, these companies have been collaborating with one another, and/or with academic groups.

- Whether the new suite of enabling technologies for PPI modulator discovery and development will enable this area to be commercially successful, or to meet the strategic needs of the industry for expanding the universe of targets for drug discovery and developing drugs that address them, remains an open question. All of the compounds that have been discovered using these technologies are in the research and preclinical stage. It will be necessary for some of these compounds to enter the clinic, achieve proof of concept, and reach the market before the value of the novel technologies can be assessed. Nevertheless, the PPI modulator field in 2012 is an exciting area that is gaining increasing interest and investment by leading pharmaceutical and biotechnology companies.

Chapter 1 Introduction

Summary

- The pharmaceutical industry is in crisis, due to falling revenues caused by the loss of patent protection of major blockbuster drugs, and the failure of R&D to produce new products that could make up for or exceed the lost revenues. Companies are responding by cutting their budgets (including R&D budgets), and by attempting to outsource more and more R&D functions, including discovery research.
- However, dependence on outsourcing of discovery research for large portions of pharmaceutical pipelines remains unproven, and simply cutting R&D investment is highly likely to decrease R&D output. To ensure companies' growth and survival, it will be necessary to directly fix the issues that have been attenuating R&D productivity.
- Recent analyses single out target selection as the key factor that is limiting the productivity of pharmaceutical R&D in the current era. The conventional wisdom is that researchers are running out of druggable targets that could be used for the discovery of novel drugs. However, as of 2011, 75% of protein research has focused on the 10% of proteins that were known before the mapping of the human genome. This is despite the finding that many proteins that were identified during the mapping of the human genome are linked to disease.
- In addition to novel druggable targets, there are emerging more challenging targets that are especially relevant to disease. Chief among these are protein-protein interactions (PPIs). Medicinal chemists have previously considered PPIs to be undruggable. However, there has been recent progress in developing small-molecule and peptide drugs designed to address PPIs. Since the human interactome includes between 130,000 and 650,000 PPIs, even if only a small fraction were relevant to disease and could be addressed by drugs, PPIs would still constitute an enormous are of unaddressed target opportunity.
- Current concepts of druggability are centered on the use of combinatorial chemistry and high-throughput screening in drug discovery, coupled with the Lipinski "Rule of Five," which predicts the "drug-like" properties of a small organic compound. This dominant paradigm has, for example, greatly diminished the role of natural products in drug discovery. The poor coverage of chemical space by

combinatorial libraries has limited researchers' ability to discover drugs for challenging targets, especially PPIs.

- The contact surfaces involved in PPIs are large and lack the types of cavities present in the surfaces of proteins that bind to small-molecule ligands. PPIs also do not have natural small-molecule ligands that can be used as starting points for drug design. Despite these challenges, focusing on "hotspots" in PPI interfaces can enable researchers to design small-molecule PPI modulatory drugs.
- There are marketed natural product drugs that modulate PPIs, such as the vinca alkaloids, colchicine, cyclosporine, and tacrolimus. Moreover, several more-recently discovered PPI inhibitors are in clinical development, and one, the thrombopoeitin mimetic eltrombopag (Ligand/GlaxoSmithKline's Promacta/Revolade), has reached the market. This indicates that it is feasible to discover and develop small-molecule PPI modulators.

The new strategic importance of protein-protein interactions

The pharmaceutical industry is in a period of crisis. Large pharmaceutical companies are being severely affected by the "patent cliff," in which blockbuster drugs that have been the mainstay of company sales lose patent protection and thus become subject to generic competition. In particular, Pfizer, the world's largest pharmaceutical company in terms of sales, is the large pharmaceutical company that is most affected by upcoming patient expirations. Patent expirations are expected to expose approximately two-thirds of Pfizer's total sales to generic competition over the next three years (Cressey, 2011). This is mainly due to Pfizer's dependence on revenues from the statin drug atorvastatin (Lipitor), which went off-patent in November 2011. Other large pharmaceutical companies, such as Novartis, Merck, Sanofi (which was until recently known as Sanofi-Aventis), GlaxoSmithKline, and Roche, also face sales losses of billions of dollars due to patent expiries over the next three years.

The exposure of large pharmaceutical companies to the patent cliff is not only caused by the loss of patent protection of leading blockbuster drugs, but by the failure of pharmaceutical R&D (including internal and partnered R&D) over at least the last decade to produce new products that could make up for or exceed the revenues from blockbusters that are losing patent protection, despite increasing R&D spending. To balance

the expected loss of drug revenues over the short term, and in particular to meet shareholder expectations regarding the market value of company stock, large pharmaceutical companies have been cutting their budgets, and in particular cutting their R&D budgets (Cressey, 2011). They are reluctant to invest heavily in R&D pipelines because these are slow to yield new drugs (as a result of long development timelines) and are high risk due to pipeline attrition. Instead, companies have been investing in buy-backs of their own stock. They are also attempting to outsource more and more R&D functions, including discovery R&D.

However, it remains unproven whether the new dependence of large pharmaceutical companies on outsourcing of discovery R&D for large portions of their pipelines will be successful, and simply cutting R&D can only decease R&D output. Therefore, to ensure companies' growth and survival, it will be necessary to directly fix the issues that are attenuating R&D productivity.

The low productivity of pharmaceutical R&D in recent years, despite (until very recently) increasing investment in R&D, is mainly due to pipeline attrition (Bunnage, 2011). Attrition is especially severe in Phase II of clinical development, with only around 25% of drugs entering Phase II proceeding through to Phase III trials. Phase II is the clinical stage in which researchers conduct "proof-of-concept" studies. These are studies aimed at supporting the clinical hypothesis that modulation of a specific biological target will have efficacy in treating a disease in humans. Since many Phase II studies assess new drugs directed at known targets, the 25% figure may be an overestimate of Phase II survival for new drugs directed at unprecedented targets (Bunnage, 2011). This suggests that a key factor – if not *the* key factor – in Phase 2 attrition is poor target selection.

Although there are several factors (ranging from target selection through drug design, preclinical studies, identification and use of biomarkers, and design of clinical trials) that can influence Phase II (and Phase III) attrition (Haberman, 2009), several recent analyses single out target selection as the key factor that is limiting the productivity of pharmaceutical R&D in the current era. In particular, Bunnage (2011) and Stockwell (2011; reviewed by Lowe, 2011) have championed this view.

Since around 2000, researchers have defined the "druggable genome" (Hopkins and Groom, 2002) as those genes that express proteins that are likely to be addressable by "drug-like" small molecules, especially those small molecules that would be likely to serve as oral drugs. Of the approximately 30,000 protein-coding

genes that were thought to constitute the human genome in 2000 (the number has since been revised downward to as low as 20,500 (Clamp et al., 2007)), only about 10% express proteins that are likely to be addressable by drug-like compounds. According to these analyses (Hopkins and Groom, 2002), if this number is combined with the number of genes that are likely to be relevant to disease, there may be as few as 600 good human drug targets. However, many of these targets have already been addressed by drugs that are on the market. According to these analyses, the number of good targets for oral drugs has been diminishing, and thus the opportunities to develop novel drugs that address unmet medical needs (as opposed to “me too” or “me better” drugs) have been rapidly disappearing.

However, according to Bunnage (2011), this is by no means true. Even among the protein families that have provided most of the targets for existing drugs, drug discovery research and even basic research has focused on a small percentage of targets. As late as 2011, 75% of protein research has focused on the 10% of proteins that were known before the mapping of the human genome (Edwards et al. 2011). This is despite the finding that many proteins that were identified after the mapping of the human genome are linked to disease. This is true even for protein families that have been central to the discovery of oral drugs, such as kinases, ion channels, and nuclear receptors. For example, among the 500 kinases in the human genome, only 10% of them have been the focus of significant research activity, even though hundreds of them have been linked to human disease,

In addition to the multitude of new targets in protein families that have been the usual targets of drug discovery, there are several families of potential targets that are generating significant interest for academic and industrial drug discovery researchers because of their disease relevance. Bunnage (2011) singles out enzymes involved in epigenetic regulation, and the proteins of the ubiquitin-proteasome system that regulates protein homeostasis.

In addition to novel “druggable targets,” Bunnage (2011) cites the importance of protein-protein interactions (PPIs) as emerging targets. As discussed later in this chapter, PPIs have been considered to be “undruggable” by medicinal chemists, despite the central importance of PPIs in biochemical pathways, including pathways important in disease processes. As discussed later in this report, there has been recent progress in developing small-molecule and peptide drugs designed to modulate PPIs. Since the human

interactome (the whole set of PPIs in human cells) is estimated to include between 130,000 and 650,000 PPIs, even if only a small fraction were relevant to disease and could be addressed by drugs PPIs would still constitute an enormous area of unaddressed target opportunity. (Note also that two other key areas of opportunity for drug discovery cited by Bunnage – epigenetic regulation and the ubiquitin-proteasome system – also involve PPIs.)

In view of the high Phase II attrition, it is well accepted that improving the quality of target selection is the single most important factor in increasing the productivity of the pharmaceutical industry. Expanding the universe of potential targets for drug discovery by including the novel targets discussed in the previous paragraphs will be essential for drug discovery researchers to identify quality targets (i.e. targets that are disease-relevant, that are addressable by drugs, and that can yield safe drugs that can provide significant improvements over current standards of care).

Stockwell goes even further in emphasizing the importance of PPIs in the future of drug discovery (Stockwell, 2011; Lowe 2011). He explains the difficulties that the pharmaceutical industry has had in recent years in developing new medicines by the industry's running out of tractable targets (i.e. targets that can readily be addressed via medicinal chemistry to produce orally available drugs). Only 2% of human proteins have been targeted with drugs as of 2011. However, most of the remaining disease-relevant proteins, such as transcription factors and many other signaling proteins, work via interacting with other proteins in PPIs. These interactions have been widely considered "undruggable." PPIs are involved in nearly every disease process, including biological pathways involved in many types of cancer, neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS)), immune/inflammatory diseases, metabolic diseases (type 2 diabetes and obesity), and many others.

If the pharmaceutical industry wishes to make progress in R&D, it needs to develop suites of tools to address PPIs and other hitherto "undruggable" targets. As discussed in this report, companies and academic groups have been active in developing these tools, and in some cases have advanced into the clinic and onto the market drugs designed to address PPIs.

As shown by the above recent publications, industry experts are recognizing the emerging strategic importance of PPIs as drug targets, and the important and perhaps crucial role of PPI drugs in reversing the productivity crisis in pharmaceutical R&D.

The new strategic importance of PPIs overlaps with the more traditional reasons for academic and industry interest in discovering drugs that address these targets. Researchers have long had a strong interest in targeting PPIs in the following classes, in terms of cellular physiology:

- PPIs in signaling pathways
- Cell-surface receptors for protein ligands
- PPIs that control apoptosis
- PPIs in the ubiquitin system.

In particular, PPIs are central to intercellular signaling pathways, which often become dysregulated in cancer, metabolic diseases, immune diseases, and other major diseases. In some cases there are no traditionally druggable targets in key signaling pathways involved in these diseases, so the ability to target PPIs would open up the possibility of combating such diseases via pathways that have not previously been feasible. PPIs are also involved in the activity of cell-surface receptors that have protein or peptide ligands. In some cases there are large-molecule drugs that serve as agonists to these receptors, such as insulin, recombinant granulocyte colony stimulating factor (G-CSF) (Amgen's Neupogen), and trastuzumab (Genentech's Herceptin). However, if small-molecule, orally available agonists could be developed, they would be more convenient for the patient, have the potential to be less expensive, and could avoid injection site reactions and immunologic side effects. The targets of currently marketed biologics are also by definition very well validated. They thus would not need to be subjected to target validation tests, which have had a low probability of success in predicting clinical efficacy. PPIs are also integral to two complex physiological processes that are especially important in cancer, but also other diseases – apoptosis and the ubiquitin system of protein homeostasis. As discussed later in this report, the ubiquitin system has recently become of special interest in drug discovery. Targeting PPIs will be important in realizing the opportunities that many researchers see in the protein homeostasis area for developing new drugs.

This report discusses progress in the discovery and development of drugs to address PPIs, including general strategies for targeting protein-protein interactions with small-molecule compounds and with peptides, and case studies of efforts to discover such drugs.

The challenges of targeting protein-protein interactions

Druggability and its limitations

In selecting targets for drug discovery, researchers have been focusing on two major issues: target validation and druggability. Target validation refers to determining that a target is critically involved in a disease pathway, and that modulating the target with a drug is likely to have a positive therapeutic effect. A druggable target is a biomolecule that can be modulated with a drug, usually using well-proven drug discovery science and technology.

Pharmaceutical and biotechnology companies develop both large-molecule and small-molecule drugs. In recent years, large-molecule drugs or biologics, including monoclonal antibody (mAb) drugs and recombinant proteins, have become increasingly important. Analyst consensus sales forecasts predict that by 2014 the six largest-selling drugs in the world will all be biologics, with most of them being mAb drugs (Reuters, 2009). . mAbs are now the fastest-growing and most successful class of biologics. The majority of the mAbs on the market are indicated for oncology and inflammatory diseases. Fueled by expanded indications and new products, mAbs have become the major growth engine of the biologics sector, and this is expected to remain the case into the future.

Large pharmaceutical companies have been working in recent years to acquire mAb drug developers and/or to develop new large-molecule drugs internally, to fill their depleted pipelines and to make up for lost revenues due to current and impending patent expirations (Allison, 2009). As discussed earlier, much of this pipeline depletion is due to the clinical attrition of many programs developing more traditional small-molecule drugs. Thus, to make up for shortfalls in small-molecule drug development, large pharmaceutical companies have adopted R&D strategies that include a greater emphasis on large-molecule drugs.

In the case of large-molecule drugs, effective drug discovery technology is derived from the molecular biology needed to develop recombinant proteins or monoclonal antibodies (mAbs) that address appropriate

targets, such as cell-surface receptors (e.g. HER2 in breast cancer and CD20 in non-Hodgkin's lymphoma) and cytokines (e.g. tumor necrosis factor-alpha (TNF-α) in inflammatory diseases such as rheumatoid arthritis). Researchers have also developed recombinant versions of therapeutic proteins such as insulin and hematopoietin, which are the targets themselves.

Several of the cell-surface receptors and growth factors that are targets of biologics are involved in PPIs. For example, the mAb bevacizumab (Genentech/Roche's Avastin) targets vascular endothelial growth factor-A (VEGF-A), a protein that activates cellular signaling pathways by interacting with VEGF receptors (VEGFRs), which are also proteins. Adalimumab (Abbott's Humira), also a mAb, targets TNF-α, a protein that exerts its physiological effects by interacting with TNF receptors. Thus the development of biologics that inhibit or activate soluble or cell-surface proteins that are involved in PPIs circumvents the druggability problems that researchers would encounter if they attempted to develop small-molecule drugs to address the relevant PPIs.

Nevertheless, some researchers and companies would like to develop small-molecule drugs to substitute for the current large-molecule drugs. Such small-molecule drugs might be orally available, and would have the potential to be less expensive than the corresponding biologics. In one case, researchers developed such a small-molecule drug, the thrombopoeitin mimetic eltrombopag (Ligand/GlaxoSmithKline's Promacta/Revolade). Eltrombopag is an agonist of the thrombopoeitin receptor, a protein that in normal physiology interacts with the protein growth factor thrombopoeitin. Eltrombopag is used to treat the rare disease chronic idiopathic thrombocytopenic purpura (ITP), which is characterized by low platelet counts. Studies have also shown that the drug is effective in increasing platelet counts in patients with cirrhosis due to hepatitis C, but it is not yet approved for this indication. Eltrombopag interacts selectively with the thrombopoeitin receptor, thus triggering intracellular signaling pathways that are normally triggered by interactions of thrombopoeitin with its receptor. We shall discuss the development of eltrombopag later in this report.

Large-molecule drugs have the disadvantage that they cannot address intracellular targets. They are usually also not orally available, and must be administered parenterally. If researchers wish to address intracellular

targets (including PPIs involved in signaling pathways, for example), and especially if they want to do so with orally available drugs, they must develop small-molecule drugs.

Small-molecule drugs have been, and still are, the mainstay of the pharmaceutical industry, despite the rise of biologics in recent decades. Orally available drugs, which are almost always small-molecule drugs, have been the focus of the industry because of their ease of administration and convenience for the patient. Orally available small-molecule drugs also are usually less expensive than parenteral drugs (especially large-molecule parenteral drugs).

The discovery of orally available small-molecule drugs typically entails the use of medicinal and combinatorial chemistry, in some cases augmented by structure-based drug design. A central goal of medicinal chemistry is the design and selection of small-molecule compounds that have "drug-like properties," that is, compounds that are likely to have the good absorption, distribution, metabolism, and elimination (ADME) properties needed for orally deliverable drugs. The "Rule of Five," a set of parameters developed by Christopher Lipinski (then at Pfizer, now on the Scientific Advisory Board of Melior Discovery (Exton, PA)) that predicts a compound's solubility and permeability, is often used (Lipinski et al., 2001).

Researchers usually define druggable targets as proteins that can be addressed by drug-like small molecules. However, natural products, including marketed drugs, often do not fit the Rule of Five criteria for drug-like compounds. Lipinski et al. (2001) stated that many natural products are delivered into cells by transporter molecules. Since the Rule of Five is based on physical and chemical properties, biological systems such as transporters that affect absorption negate the Rule in the case of these natural products. Small-molecule compounds that are delivered parenterally (e.g. many oncology drugs) may also violate the Rule of Five, which is centered on the characteristics of orally available drugs.

As of 1990, some 80% of marketed drugs were either natural products or analogs based on natural products (Li and Vederas, 2009). Beginning in the 1990s, and continuing to the present day, pharmaceutical companies deemphasized natural products, and in some cases stopped pursuing natural products altogether. This is due to the ascendancy of two waves of technology-driven drug discovery: combinatorial chemistry combined with high-throughput screening (HTS) since the early 1990s, and genomics-driven drug discovery since 2000. As the result of these changes in strategy, HTS of synthetic compound libraries

against genomics-derived targets has been the governing paradigm of small-molecule drug discovery since 2000. Nevertheless, natural products and natural-product derivatives still accounted for around 50% of newly approved drugs between 2005 and 2007. Over 100 natural products and natural product derivatives were in clinical studies as of 2009.

Pharmaceutical companies and their researchers have been emphasizing organic compounds that can be synthesized using combinatorial chemistry over natural products because they are more amenable to use in HTS. These compounds have relatively simple structures as opposed to the complex structures of natural products. Natural products are typically present as small amounts of active compound in complex mixtures, making it difficult for researchers to characterize them. Once characterized, natural products are also often difficult to synthesize. Thus the paradigm for drug discovery has become combinatorial chemistry to produce libraries of small organic compounds, which are subjected to HTS to discover compounds that address targets derived from genomics. (However, as discussed earlier, researchers have still been mainly screening against targets known before the mapping of the human genome.)

The use of combinatorial chemistry and HTS allows researchers to examine large numbers of compounds in a short amount of time. This appeared to fit well with the desire of pharmaceutical companies to rapidly discover multiple drug candidates, among which may be the few drugs that could not only reach the market, but also achieve blockbuster status (i.e. $1bn per year in sales or more).

However, this strategy has not served pharmaceutical company business goals well. Synthetic chemical libraries have low hit rates – typically less than 0.001%. In contrast, for one type of natural products – polyketides – approximately 7,000 known structures have led to over 20 marketed drugs, constituting a hit rate of 0.3% (Li and Vederas, 2009). Nevertheless, pharmaceutical companies still prefer performing HTS of massive combinatorial libraries of synthetic compounds. Although hit rates are extremely low, any hits are usually easy to synthesize and modify, to develop optimized leads and eventually clinical candidates.

However, the combinatorial libraries of synthetic compounds that are commonly used by the pharmaceutical industry limit the ability of researchers to address target classes that are beyond the range of targets that are addressed by currently marketed drugs (Bauer et al., 2010). In the majority of cases these libraries are based on structural modification of existing drugs. As a result, such libraries are biased toward classes of

targets similar to those addressed by existing drugs, and they render other classes of targets, especially PPIs, undruggable. Not only is the number of targets addressed by current and experimental drugs a small fraction of the universe of targets as discussed previously, but the classes of target are also severely limited. Half of all current drugs address proteins in four classes: rhodopsin-like G-protein coupled receptors (GPCRs), nuclear receptors, and voltage- and ligand-gated ion channels (Bauer et al., 2010). Other factors, such as ease of synthesis, desirable physicochemical properties, and companies' intellectual property positions, further limit the range of chemical structures found in combinatorial libraries. It has been estimated that only about 10–14% of human proteins are druggable using current libraries of drug-like molecules. Other targets are considered "challenging targets," "hard targets," or simply "undruggable."

Combinatorial chemistry is a superb technology for optimizing the structures of lead compounds once an active skeleton has been identified, and it has been used in the optimization of many drugs approved in recent years. However, the use of current combinatorial libraries of synthetic organic molecules for *de novo* drug discovery has been sorely lacking. In a survey of all agents approved between January 1981 to June 2006, Newman and Cragg of the US National Cancer Institute were able to identify only one approved new chemical entity (NCE) that resulted from *de novo* discovery via combinatorial chemistry. (Newman and Cragg, 2007) This is sorafenib (Bayer's Nexavar), a multi-kinase inhibitor that is approved for the treatment of kidney and liver cancer. In contrast, the same authors found that natural products have played a dominant role in the discovery of drug leads, even in recent years.

Moreover, even current synthetic libraries are based on natural products (Bauer et al., 2010). Commercially available drugs and libraries exhibit much higher structural similarity to natural products and metabolites than the complete set of all possible molecules in the universe of small synthetically-accessible organic compounds. Researchers hypothesized that the reason these libraries have any effectiveness at all in identifying new small-molecule drugs is that they are "unintentionally" based on structures in naturally occurring molecules, which have coevolved with proteins and can bind them. However, since combinatorial libraries and newer drugs are based on older approved drugs, many of which were derived from natural products, the resemblance of these libraries and newer drugs to natural products may not really be unintentional.

A goal of medicinal and combinatorial chemistry would then be to develop new libraries that are based on natural product scaffolds that are underrepresented in current libraries. Studies indicate that 83% of small natural product scaffolds and 20% of small metabolite scaffolds are not represented in commercially available libraries. (The number of unrepresented scaffolds is probably higher for larger molecules, but the Rule of Five calls for focusing on compounds of less than 500 daltons with no more than five hydrogen-bond donors and no more than 10 hydrogen-bond acceptors.) Libraries that are based on these underrepresented scaffolds may enable researchers to address what are now considered “undruggable” targets.

As discussed later in this report, drugs or drug candidates that modulate PPIs include natural products or natural product-like compounds, and screening natural product libraries or synthesizing natural product-like compounds is one approach to discovering drugs that address PPIs.

Theoretical reasons for the “undruggabilty” of protein-protein interactions

There are several theoretical reasons for why PPIs represent challenging targets for small-molecule drugs, especially drug-like compounds that have the potential for oral availability (Wells and McClendon, 2007). The contact surfaces involved in PPIs are large (approximately 1,500–3,000 Angstroms) compared with those involved in interactions between proteins and small molecules (approximately 300–1,000 Angstroms). Contact surfaces in PPIs are usually flat, and lack the types of cavities present in the surfaces of proteins that bind to small-molecule ligands. Unlike the types of targets such as enzymes and GPCRs that are the targets of existing drugs, PPIs do not have natural small-molecule ligands. Thus, researchers cannot start from such natural ligands to design drug molecules.

Exacerbating the challenge, contact surfaces in PPIs (except in a very few cases) involve non-contiguous amino acid residues. In other words, these residues are not adjacent to one another in the sequence of the polypeptide chain, but are brought together via three-dimensional folding in the native structure of the protein. This makes it impossible in most cases to use short peptide chains derived from the protein structure as starting points for the design of peptidomimetic drugs. Finally, high-throughput screening using combinatorial libraries does not identity compounds that address PPIs, except in a very few cases. This might be expected for the limitations of such libraries that were discussed previously.

Overcoming the theoretical challenges to PPI druggability

Many researchers who work on the discovery of drugs that address PPIs postulate that, despite the above challenges, it may be possible to discover small-molecule drugs that modulate PPIs by focusing on "hotspots" (Moreira et al., 2007). Hotspots have been defined by mutational studies showing that in the large, generally flat binding interfaces between proteins only a small subset of amino acid residues contribute most of the free energy of binding (Wells and McClendon, 2007; Moreira et al., 2007). In some cases, researchers have investigated hotspots further via structural studies.

Hotspots are usually found in the center of a PPI. The hotspot on one face of a PPI usually interacts with a hotspot on the other face. Hotspots form complementary pockets, in which a pocket on one face of a PPI can interact with residues in a hotspot on the other face, thus contributing to tight binding between the two protein partners. These interacting hotspots are potential targets for drug discovery.

In addition to these theoretical considerations for the discovery of drugs that can address PPIs, a few marketed natural product drugs have long been known to modulate certain PPIs. For example, the vinca alkaloids and colchicine inhibit the polymerization of tubulin, and cyclosporine and tacrolimus (FK506) catalyze the formation of PPIs – specifically the interaction between immunophilin and calcineurin (Arkin and Wells, 2004). More recently, two microbial natural products, FR901464 and pladienolide B, were identified as inhibitors of spliceosomes, complexes of small nuclear RNA (snRNA) and protein subunits that remove introns from transcribed pre-mRNAs in the nucleus and splice together the adjacent coding segments of an mRNA. These compounds appear to function by inhibiting interactions between proteins in the spliceosome complex (Bauer et al., 2010).

The ability of these natural products to modulate PPIs suggests that it is possible to discover other PPI-modulating drugs (whether other natural products or newly synthesized compounds). As we shall discuss in this report, academic and corporate researchers have been working to develop technologies for the discovery of PPIs, and the number of tool compounds and drug candidates that modulate these targets has been growing in the past several years. Tool compounds, also known as chemical probes, are high-affinity, selective, and cell-permeable small molecules that perturb a biological target, and can thus be used to study the role of the target, including a PPI, in biology (Nature Chemical Biology editorial, 2010). Tool compounds

may not be good drug candidates (for example, if they do not fit the Rule of Five), but may in some cases serve as leads.) Most such drug candidates are in the research or preclinical stage. However, several others are in clinical development, and one, the thrombopoeitin mimetic eltrombopag (Ligand/GlaxoSmithKline's Promacta/Revolade), which was discussed earlier, has reached the market. This report discusses strategies for drug discovery and development in this area, as well as case studies in the discovery and development of small-molecule and peptide drugs designed to modulate PPIs.

Table 1 summarizes both the theoretical challenges to discovering small-molecule drugs that modulate protein-protein interactions, and potential means to overcome them.

Table 1: Challenges and solutions in developing drugs that target PPIs

Challenge	Ways to overcome them
Contact surfaces involved in PPIs are large, usually flat, and lack cavities present in the surfaces of proteins that bind to small-molecule ligands.	Via mutational and structural studies, researchers have identified hotspots within PPI binding interfaces. These hotspots can be potential targets for drug discovery.
PPIs do not have natural small-molecule ligands. Thus, researchers cannot start from such natural ligands to design drug molecules.	Use of such methods as fragment-based drug discovery and structure-based drug design, as discussed in Chapter 2.
PPI contact surfaces usually involve non-contiguous amino acid residues. Thus one cannot use short peptide chains derived from the protein structure as starting points for the design of peptidomimetic drugs.	A few PPIs do have contact surfaces that utilize contiguous amino acid residues for binding between the proteins. Researchers can use these to design peptidomimetic drugs.
High-throughput screening using combinatorial libraries does not usually identity compounds that address PPIs.	Most libraries are biased toward targets of existing drugs. Use natural product or natural product-like libraries, or libraries that include underrepresented natural product-like scaffolds.

Source: Haberman Associates

INFORMA

Structure of this report

Chapter 2 discusses general strategies for targeting protein-protein interactions with small-molecule compounds. Chapters 3–6 discuss cases studies in the discovery and development of small-molecule compounds that target PPIs: those that target cell-surface receptors (Chapter 3), those that target

intracellular signaling pathways (Chapter 4), and those that target the ubiquitin system (Chapter 5) and PPIs that control apoptosis (Chapter 6). Chapter 7 discusses the discovery and development of stapled peptides designed to target PPIs. These are peptides in which hydrocarbon moieties are used to constrain, or “staple,” peptide sequences that are designed to interact with key domains of protein targets. Chapter 8 outlines the conclusions of the report, and discusses the outlook for the field of small-molecule and peptide drugs that modulate PPI targets.

Chapter 2 General strategies for targeting protein-protein interactions with small molecules

Summary

- Researchers have developed a set of tools for discovering small-molecule drugs that target PPIs, which have been used to discover all the compounds that have so far reached the clinic. These include alanine scanning mutagenesis, X-ray crystallography, and such fragment-based drug design (FBDD) methods as tethering and structure-activity relationship by nuclear magnetic resonance (SAR by NMR). These have been combined with such methods as screening of chemical and natural-product libraries using cell-based assays.
- This set of technologies has not been powerful enough to enable the regular and consistent discovery of small-molecule PPI modulators. Compounds have so far been discovered on a “one compound at a time” basis.
- However, in recent years researchers have developed new technologies that have the potential to move this field up the technology development curve and enable the regular discovery of small-molecule PPI modulators.
- A key innovation is computational solvent (CS) mapping, which enables the virtual identification of druggable binding sites within PPIs, and the subsequent discovery or design of small-molecule PPI modulators.
- Another important advance has been in the design of improved chemical libraries for targeting PPIs, via such methods as diversity-oriented synthesis (DOS) and the synthesis of macrocyclic compounds via Ensemble Therapeutics’ DNA-programmed chemistry (DPC) technology.
- These new technologies are combined with cellular screening assays (including high-content screening) and medicinal chemistry to discover or design PPI modulators.

- Ensemble Therapeutics and Forma Therapeutics are particularly prominent in applying PPI-focused technologies. Both have discovered preclinical-stage small-molecule PPI inhibitors, and both have attracted alliances with big pharma companies, specifically Novartis, Pfizer, and Bristol-Myers Squibb.
- Companies had previously been abandoning or cutting back on their PPI modulator discovery efforts. However, as a result of the development of the new suite of enabling technologies, both venture capital investment and companies – including big pharma – have been moving back into PPI discovery. Several other companies are continuing to develop compounds that resulted from earlier now discontinued or reduced PPI modulator discovery programs (e.g. GlaxoSmithKline (in collaboration with Ligand), Roche, and Abbott).
- With the new strategic importance of PPIs as targets, we expect additional companies to enter the field.

Introduction

This chapter discusses general strategies and technologies for the discovery of small-molecule drugs that target PPIs. This will serve as a foundation for the discussion in Chapters 3–6 of case studies of the discovery and development of small-molecule compounds that target particular PPIs. This chapter also includes a discussion of a set of new technologies designed to move the field up the technology development curve and enable the regular discovery of small-molecule PPI modulators.

Structural biology studies to determine “hotspots” in protein-protein binding interfaces

As was discussed in Chapter 1, despite the large sizes of interfaces in PPIs, only a small subset of amino acid residues contributes most of the free energy of binding. These “hotspots” are potential targets for drug discovery. Thus an important strategy in designing or discovering small-molecule modulators of PPIs has been the structural determination of hotspots.

A classic way of identifying and defining hotspots in PPIs, going back to the mid-1990s (but still being used to this day), has been the combination of X-ray crystallography and alanine scanning mutagenesis (Moreira et al., 2007). The initial application of this strategy, in 1995, was by Timothy P. Clackson and James Wells,

who identified a hotspot in the binding interface between human growth hormone (hGH) and the extracellular domain of its receptor (hGHbp) (Clackson and Wells, 1995). (This work was done at Genentech. Clackson is now at Ariad, and Wells is now at the University of California at San Francisco (UCSF).)

In that study, X-ray crystallography showed that in the interface between hGH and hGHbp about 30 side chains from each of the two proteins make contact. However, individual replacement of each of these side chains with alanine showed that a central hydrophobic region, containing two critical tryptophan residues, contributed over 75% of the binding free energy of the PPI.

The rationale for using alanine substitution in this technology is that alanine replacement eliminates the amino acid side chain beyond the beta-carbon. However, it does not alter the conformation of the main polypeptide chain (as glycine or proline can), nor does it introduce novel steric or electrostatic effects (Cunningham and Wells, 1989). Since alanine scanning mutagenesis requires the production of a new, correctly-folded protein for each amino acid residue to be substituted with alanine, it is a slow, laborious process.

Using alanine scanning mutagenesis, researchers have identified hotspots for numerous PPIs (Wells and McClendon, 2007). These hotspots make up less than half of the contact surface of a PPI, but contribute the majority of the binding free energy of the PPI.

Some protein regions that are involved in PPIs are “promiscuous” (Moreira et al., 2007; Wells and McClendon, 2007). These regions bind to different partners, using the same hotspot. Structural studies show that in these promiscuous contact surfaces the same hotspots are adaptable, allowing the promiscuous binding region to engage sets of structurally diverse partners. For example, the Fc fragment of immunoglobulin G (IgG) (and of other classes of antibodies) interacts with cell-surface receptors known as Fc receptors on such cells as macrophages, neutrophils, natural killer cells, and mast cells. When these effector cells, via their Fc receptors, bind to antibodies that are attached to virus-infected cells or invading pathogens, this triggers the effector cells to destroy the infected cells or pathogens via such processes as antibody-mediated cellular cytotoxicity or phagocytosis.

The hinge region of the IgG Fc fragment not only binds to Fc receptors, but also to Protein A (a *Staphylococcus aureus*-derived protein that is used in industrial purification of antibodies), Protein G (a streptococcal-derived antibody-binding protein), and rheumatoid factor (an autoantibody directed against IgG Fc) (DeLano et al., 2000). The same small domain of the Fc fragment (a site between the CH2 and CH3 domains) interacts with all these partners, even though the partners are structurally diverse. This binding site is an exposed hydrophobic region that contains relatively few amino acid residues capable of polar interactions. Using phage display technology, researchers selected for random peptides that showed high affinity binding to the Fc fragment. These peptides also preferentially bound to same small domain of the Fc fragment.

Alanine scanning mutagenesis demonstrated that five amino acid residues in the Fc fragment hinge region binding domain were critical for binding of partner proteins or peptides. Alanine replacement of residues on partner proteins or peptides also showed that only a few amino acid residues are critical for binding of the partner. Thus the Fc fragment hinge region binding domain constitutes a classic hotspot. This hotspot is thus not only an energetically important site, but also an adaptive region that is capable of interaction with a variety of structurally diverse partner molecules. The ability of researchers to select for peptides that bind to this hotspot suggests that it is a preferred binding site on Fc. Other domains involved in PPIs also possess preferred binding sites.

Insights from the study of interleukin-2 (IL-2) and its binding to the IL-2 receptor alpha chain (IL-2Rα)

Mutagenesis and structural studies

Interleukin-2 is a cytokine that, via binding to its receptor, stimulates T-cell proliferation. It is a critical component of the immune system in health and disease. Immunosuppressive drugs such as corticosteroids, cyclosporine, tacrolimus, and sirolimus work on intracellular signaling pathways either to inhibit production of IL-2 or to inhibit T-cell responses to IL-2. However, especially in the 1990s and early 2000s, researchers were seeking to discover and develop both large and small-molecule drugs aimed at modulating IL-2 activity. In the case of small-molecule drugs, the researchers were specifically aiming to develop compounds that address the PPI between IL-2 and IL-2Rα. This work was reviewed by Wilson and Arkin (2011).

With respect to large-molecule compounds, two similar mAbs to IL-2Rα have been developed: dadizumab (Roche's Zenapax) and basiliximab (Novartis's Simulect). Basiliximab is approved by the FDA to prevent kidney transplant rejection. Dadizumab is in Phase II trials in the US for kidney transplant rejection. It has been approved for the same indication in Europe. However, at the request of the manufacturer, marketing authorization for dadizumab was withdrawn in 2009, because of diminishing market demand.

Basiliximab and, formerly, dadizumab are used during the acute phase of and for the prevention of transplant rejection; they are not used for the long-term maintenance of immune suppression in transplant patients. These mAb drugs are expensive, require parenteral administration, are cleared from the body slowly (which complicates dosing), and eventually lose their efficacy owing to the body's production of anti-drug antibodies. Moreover, there are less expensive, small-molecule immunosuppresants that are effective in most transplant patients, although these immunosuppressants come with their own set of often-severe adverse effects.

In the 1990s and early 2000s, researchers at Roche and at Sunesis (South San Francisco, CA), a company that at that time was focused on PPI modulators, were working to identify and characterize hotspots and to develop small-molecule inhibitors of the IL-2/IL-2Rα interaction. (More recently, Sunesis has changed its focus to the development of oncology drugs that do not work via PPI inhibition. As will be discussed in Chapter 3, Sunesis outlicensed one of its PPI programs to SARcode, which is continuing the development.) Such small-molecule IL-2Rα inhibitors were envisioned to have applications not only in organ transplantation, but also other indications in which IL-2 was central, such as autoimmune diseases. As discussed by Wilson and Arkin (2011), Sunesis researchers (building on initial research at Roche) made considerable progress in developing small-molecule IL-2Rα inhibitors. Eventually, the researchers developed lead compounds with nanomolar affinity (Raimundo et al., 2004). However, Sunesis's doubts about the clinical and commercial value of IL-2Rα antagonists resulted in the decision to exit the program before the researchers could assess whether they could create a compound that had *in vivo* activity (Wilson and Arkin, 2011).

Nevertheless, the technologies and strategies developed for identifying and characterizing hotspots and lead modulators of the IL-2/IL-2Rα PPI, and the lessons learned in carrying out these early programs, have been applicable to later studies aimed at developing PPI modulators. The researchers used a combination of

structure-based design, fragment-based drug discovery, and medicinal chemistry; this type of strategy has been applicable to later programs.

In 1991, Roche researchers used site-specific mutagenesis (which included replacement of native amino acid residues with alanine as well as other amino acids) to determine the binding site on IL-2 that interacts with IL-2Rα (Sauvé et al., 1991). This site was in a concave, mainly hydrophobic face of IL-2, and contained four surface residues (lysine 35, arginine 38, phenylalanine 42, and lysine 43) that were critical for receptor binding. This site thus constitutes a typical PPI hotspot.

Academic researchers later determined the structure of the IL-2/IL-2Rα complex (Wilson and Arkin, 2011). These studies confirmed the IL-2 hotspot, identified the corresponding IL-2Rα hotspot, and showed how the two hotspots interact with each other in the PPI interface. The interface has an area of about 1,900 Angstroms, and consists of 20 IL-2 amino acid side chains and 21 IL-2Rα side chains. The residues contributed by each protein are derived from sequential and nonsequential portions of their primary sequences, as is typical for PPIs.

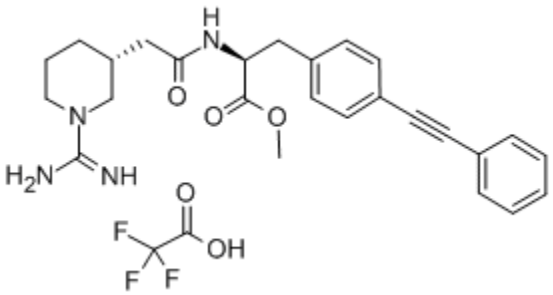
The two interacting hotspots within the interface are composed of hydrophobic patches, including IL-2 side chains phenylalanine 42 and leucine 72, projecting into a complementary cavity formed by leucine 42, tyrosine 43, and methionine 25 on the surface of IL-2Rα, and a buried salt-bridge between glutamic acid 62 of IL-2 and arginine 36 of IL-2Rα. (A salt-bridge is a combination of a hydrogen bond and an electrostatic interaction, typically between the carboxyl group of an acidic amino acid and the amino group of lysine or the guanidinium group of arginine both of which are basic groups. It is thus a type of non-covalent interaction between an acidic and a basic amino acid residue that contributes stability to the folded structure of a protein.) There are numerous polar and salt-bridge interactions surrounding the interacting hotspots.

Ro26-4550 and the nature of the IL-2/IL-2Rα hotspot

Ro26-4550, the first small molecule capable of inhibiting the IL-2/IL-2Rα interaction, was discovered serendipitously by Roche researchers in 1997 (Wilson and Arkin, 2011). The researchers designed acylphenylalanine analogs to mimic the binding region of IL-2 (as identified by site-specific mutagenesis), and therefore to bind to IL-2Rα. However, these compounds bound not to IL-2Rα, but to IL-2 itself. One of these compounds, Ro26-4550, (Figure 1) was studied further at Roche and eventually at Sunesis (Wilson

and Arkin, 2011). As shown in Figure 1, Ro26-4550 contains a hydrophobic biaryl alkyne group (i.e. the two benzene rings joined to each other by the triple-bonded carbons) at one end of the molecule, and a basic, hydrophilic piperidyl guanidine group at the other end. The compound was a competitive inhibitor of IL-2Ra, with a modest half-maximal inhibitory concentration (IC_{50}) of 3μM. The compound was thus not a drug candidate. However, it was the first small-molecule PPI inhibitor to be discovered, and thus provided proof-of-principle that small-molecule PPI inhibitor drugs might be feasible to be discovered or designed.

Figure 1: Chemical structure of Ro26-4550



Source: www.chemicalbook.com

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Moreover, structural studies at Sunesis of Ro26-4550 bound to IL-2 helped to characterize the IL-2 binding site, and served as the starting point for the design of higher-affinity small-molecule IL-2/IL-2Rα PPI inhibitors.

The structure of IL-2 with bound Ro26-4550, determined via X-ray diffraction, displays striking differences in protein conformation from unbound IL-2. This finding contrasts with the structure of IL-2 bound to IL-2Rα, in which the structure of IL-2 appears unchanged. Although unbound and IL-2Rα-bound IL-2 presents a flat surface, Ro26-4550 binds in a groove on IL-2 formed primarily by rearrangements of surface side chains, especially phenylalanine 42, as well as slight movement of the area of the main chain associated with lysine 35. The groove buries around 60% of the surface area of Ro26-4550. The biaryl alkyne group at one end of Ro26-4550 is enclosed in the hydrophobic pocket that is formed by the IL-2 hotspot that had been determined via site-specific mutagenesis as mentioned earlier, and is also critical for IL-2 interaction with IL2Ra.

A second, acidic subsite on the IL-2 molecule is centered on glutamic acid 62. Glutamic acid 62 forms a salt-bridge with the piperidyl guanidine group of Ro26-4550 at the other end of the molecule. This basic group on Ro26-4550 thus acts as surrogate for the guanidinium group of arginine 36 of IL2-Rα. Unlike the hydrophobic pocket, the acidic subsite does not undergo significant conformational change.

The structural studies with IL-2 bound to Ro26-4550 showed that the area of the hotspot on the surface of IL-2 that interacts with IL-2Rα is dynamic and can accommodate selective small-molecule compounds. It also illumines the nature of the hotspot, which consists of hydrophobic sub-sites and an acidic sub-site. The dynamic, adaptive nature of the IL-2 hotspot is reminiscent of the Fc domain hotspot that was discussed earlier, and it also applies to other PPI hotspots. The Ro-26-4550 studies also suggested that researchers should be able to design small-molecule inhibitors of at least some PPIs by exploiting the adaptivity of hotspots in PPI interfaces.

Fragment-based discovery of small-molecule PPI-modulating drugs

Fragment-based drug discovery (FBDD) (which, since it is initially aimed at the design or discovery of lead compounds, is also known as fragment-based lead discovery (FBLD)) is a key strategy for the discovery and design of small-molecule modulators of PPIs, although it is more often applied to the discovery of drugs to modulate other types of targets, such as kinases. In general, FBLD involves first identifying small chemical fragments (typically around 200 Daltons), which may only bind at millimolar affinity to their targets. Researchers then expand the fragments or link them to other fragments that bind to nearby regions on the target, to design a lead with stronger affinity (Everts, 2008). These leads are then optimized via medicinal chemistry, and may then be entered into preclinical and eventually clinical studies.

FBLD may result in the discovery of lead compounds that do not exist in corporate compound files or in libraries produced by combinatorial chemistry, and that are difficult to produce by conventional methods. Among these novel compounds are potential modulators of PPIs and other challenging targets.

This section includes a discussion of two methods for FBLD/FBDD of potential modulators of PPIs: tethering and structure-activity relationship by nuclear magnetic resonance (SAR by NMR).

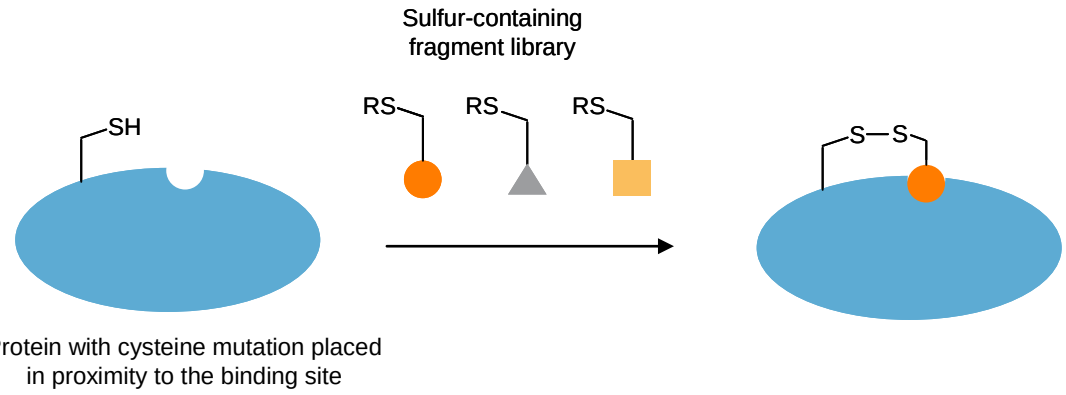
Tethering

Sunesis researchers developed a technology, known as tethering, for fragment-based drug discovery of small-molecule PPI-modulating drugs (Wilson and Arkin, 2011; Arkin et al., 2003), and they used this technology to discover inhibitors of the IL-2/IL-2Rα PPI. As will be discussed, the results of these studies have implications for the field of small-molecule modulators of PPI in general, and especially for the discovery and design of small-molecule PPI modulators via fragment-based drug discovery.

To carry out FBLD via tethering, researchers first construct mutant forms of the target protein that contain the amino acid cysteine near a domain involved in a PPI. In the case of the first protein for which this was carried out, IL-2, Sunesis researchers targeted the region of the binding site for Ro26-4550, which had also been determined to be a hotspot via site-directed mutagenesis as discussed earlier. One limitation of the application of tethering is that it requires identification of such an appropriate site.

In parallel, the researchers construct a fragment library of organic compounds of less than 200 Daltons in molecular weight, each of which is linked to a disulfide moiety. The researchers then expose that library to the protein, under conditions that promote thiol exchange. The goal is to select for compounds that bind to the PPI binding site near a native or engineered cysteine residue. Cysteine contains a thiol (SH) group that is capable of linking with another SH group to form a disulfide linkage. If a fragment has affinity for the site on the protein, it becomes linked to the cysteine by a disulfide bond. This is illustrated in Figure 2. This binding can be detected by mass spectrometry. Because the reversible disulfide bond stabilizes the binding of the fragment to the protein site, tethering can be used to identify fragments that bind weakly to a protein site.

Figure 2: Use of tethering to identify a lead in fragment-based drug design



Source: Haberman Associates

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To form lead compounds, different fragments that bind to neighboring sites are linked using a stable, non-disulfide linker. These can then be optimized using the methods of medicinal chemistry to produce lead compounds.

The Sunesis researchers used tethering to explore the IL-2 binding site for Ro26-4550 (Wilson and Arkin, 2011; Arkin et al., 2003). They first produced a series of functionally benign cysteine mutants of IL-2 around the binding site. They then screened each of the mutants with a large tethering library (i.e. a library of small molecules linked to a disulfide moiety). The cysteine mutants differed in the numbers of compounds that became tethered to the protein. The cysteine with the most hits was in the adaptive hydrophobic sub-site, and it bound hydrophobic tethering compounds that were similar chemically to the hydrophobic moiety of Ro26-4550. Structure determination via X-ray diffraction showed that one of the strongest hits results in a similar protein conformation within the sub-site to Ro26-4550, without affecting the acidic sub-site.

Tethering compounds that bound to the acidic sub-site had strict requirements for chemical structure. They all terminated in a guanidine group, such as is found in Ro26-4550, and had strict requirements for the length of the spacer between that group and the sulfhydryl group that allowed for tethering to the mutant cysteine residue. X-ray diffraction studies showed that one of the strongest hits formed some of the same interactions with the protein sub-site as does Ro26-4550, especially the salt-bridge with glutamic acid 62.

These tethering studies confirmed the nature of the IL-2 hot spot, as consisting of an adaptive hydrophobic sub-site and a rigid hydrophilic sub-site. Compounds that would be capable of binding to the site with high affinity must bind to both sub-sites. The mobile, adaptive nature of the hydrophobic sub-site makes it difficult to use X-ray diffraction of unbound IL-2 to design small-molecule ligands via structure-based drug design. As discussed earlier, many other PPI interfaces are also dynamic and adaptive. This is one reason for the challenging nature of PPIs as targets for small-molecule drug discovery. However, adaptive binding sites can provide grooves or cavities for binding small molecules, which may be exploited by drug discovery researchers if they can develop methodologies to accomplish this.

The Sunesis researchers built on these initial studies to design a higher-affinity IL-2Rα receptor antagonist via a combination of medicinal chemistry and tethering, using Ro26-4550 as a starting point (Wilson and Arkin, 2011; Raimundo et al., 2004). This research resulted in the discovery of a higher affinity (60nM binding constant) IL-2Rα receptor antagonist known as SP4206.

Initial studies of SP4206 as a drug lead were favorable (Wilson and Arkin, 2011; Raimundo et al., 2004). The compound bound to mouse IL-2R on the surface of a cell line and activated intracellular signaling. SP4206 also showed favorable pharmacokinetic properties in mice. However, Sunesis's doubts about the value of IL-2Rα antagonists as human therapeutics led to the abandonment of the program before the researchers could take lead optimization and preclinical development further. And, as discussed previously, Sunesis changed its focus to the development of oncology drugs that do not work via PPI inhibition. The company has continued to use tethering in drug discovery in non-PPI applications. One of Sunesis's clinical candidates, the Aurora kinase inhibitor SNS-314 (now in Phase I clinical trials), was discovered via use of tethering. Moreover, as will be discussed in Chapter 3, Sunesis compounds for modulation of the PPI between lymphocyte function-associated antigen 1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) were outlicensed to SARcode, and are being developed by that company.

SAR by NMR

SAR by NMR is a FBLD/FBDD method that was developed by Stephen Fesik and his colleagues at Abbott Laboratories (Shuker et al., 1996). In 2009, Fesik moved to Vanderbilt University (Nashville, TN), where he leads cancer drug discovery research at the Vanderbilt Institute of Chemical Biology and the Vanderbilt-

Ingram Cancer Center. (This represents yet another leader in small-molecule PPI modulator discovery, along with James Wells, who has moved from industry to academia.) At Vanderbilt, Fesik and his colleagues work on cancer drug discovery via FBDD and structure-based design. The emphasis is on highly validated but challenging or "undruggable" targets like K-Ras and c-Myc.

In SAR by NMR, researchers use a high-throughput NMR technique to screen chemical libraries for fragment-sized compounds that bind to a protein sub-site with micromolar binding constants. Since researchers can use structural information from NMR to locate the binding sites for the compounds within a sub-site, they can identify ligands that bind to distinct but nearby sites within the sub-site. They then can link the two ligands together to produce a new compound that binds to the domain with high affinity. This compound can be further optimized via medicinal chemistry to give lead compounds and ultimately drug candidates.

The Fesik group at Abbott, in collaboration with Idun (San Diego, CA) and with academic collaborators, applied SAR by NMR to the discovery of inhibitors of B-cell lymphoma-2 (Bcl-2) family members that inhibit apoptosis (Oltersdorf et al., 2005). Bcl-2 family members include proapoptotic and antiapoptotic members, which interact with each other by means of specific PPIs; thus these compounds are PPI modulators that act to promote apoptosis in certain cancers. Pfizer acquired Idun in 2005, and in 2011 Conatus Pharmaceuticals (San Diego, CA) acquired Idun from Pfizer.

The Fesik group's research eventually led to the discovery of the Bcl-2 inhibitor ABT-263, also known as Navitoclax, which works via stimulating apoptosis in tumors. As the result of Abbott's selection of a development candidate during the Idun collaboration, it retained the rights to the compounds that target the Bcl-2 family that came out of this research. However, Conatus has rights to milestone and royalty payments as Navitoclax progresses through clinical trials. Abbott is currently codeveloping Navitoclax with Genentech. Navitoclax is in Phase I and Phase II clinical trials in various cancers, including combination therapies with such Genentech drugs as Rituxan (rituximab) and Tarceva (erlotinib), as well as with cytotoxic chemotherapies and as a single agent.

The discovery and development of the Fesik group's Bcl-2 family modulators is discussed in further detail in Chapter 6.

Computational identification of hot spots for fragment-based drug design

Small-molecule modulators of PPIs so far have been discovered on a “one compound at a time” basis, rather than by application of a powerful suite of technologies and strategies to enable the regular and consistent discovery of such compounds. Thus the material in Chapters 3–6 of this report consists of a collection of case studies, each one of which describes the discovery and in some cases the development of such compounds.

The discovery of compounds to modulate PPIs to date thus has represented a “premature technology” or, in other words, a field of biomedical science in which, despite the exciting potential of the field, consistent practicable therapeutic applications are in the future, due to difficult technological hurdles (Haberman, 2009a). Perhaps the classic example of a premature biomedical technology was the field of mAb drugs between 1975 (the discovery of mAb technology) and the mid-1990s. Despite the exciting and Nobel Prize-winning science of mAbs, and despite the ready application of mAb technology to basic research, the consistent development of mAb-based therapeutics required the development of a suite of enabling technologies, specifically the development of chimeric, humanized, and fully human mAbs that enabled the production of minimally immunogenic MAbs with the potential for efficacy and safety in humans (Haberman, 2009b). Currently, mAb drugs represent one of the most successful classes of therapeutic products.

In addition to small-molecule PPI modulators, other contemporary examples of premature biomedical technologies are RNA interference (RNAi)-based drugs and stem cell-based therapeutics (Haberman, 2009a).

As in the case of mAb drugs, the field of small-molecule PPI modulators requires the development of a set of enabling technologies to drive the field up the technology development curve and render the discovery and development of these drugs routine or at least fairly commonplace. The technologies and strategies discussed to this point in this chapter represent a set of enabling technologies, and these have not been robust enough to enable the regular development of small-molecule PPI modulators. Several of the technologies discussed in the rest of this chapter are novel, and are aimed at moving the field of small-

molecule PPI modulator discovery and development up the technology curve, thus enabling the regular development of small-molecule PPI modulators.

The work of Sandor Vajda and his colleagues at the Structural Bioinformatics Laboratory in the Department of Biomedical Engineering at Boston University (Boston, MA) has been aimed at developing computational methods for the modeling of PPIs (especially hotspots) and for drug design. In 2004, Vajda and his colleagues founded SolMap Pharmaceuticals (Cambridge, MA), a start-up company that applied computational methods to FBDD. In late 2005, Mercury Computer Systems (Chelmsford, MA) invested in SolMap, providing the necessary computer technology to increase SolMap's computational power (using an STI (Sony/Toshiba/IBM) Cell (cell broadband engine architecture) processor, which is used for example, in Sony's PlayStation 3 (Davies, 2008)) and becoming SolMap's parent company.

In 2008, Forma Therapeutics (Watertown, MA; Forma moved from Cambridge, MA, in late December 2011) purchased the assets and intellectual property of SolMap from Mercury. Forma is working on the development of small-molecule PPI inhibitors, and we shall discuss this company's technology strategy later in this chapter. Meanwhile, Vajda and his colleagues went on to form a new company, Acpharis (Boston, MA). Acpharis works on software development and consulting in computational chemistry and biology, with a focus on PPIs.

In 2011, Vajda and coworkers published a research report describing computational methods for identifying sites within PPI interfaces that are capable of binding drug-sized (as opposed to fragment-sized) ligands (Kozakov et al., 2011). Such an approach might be extremely useful in the discovery and design of lead compounds that address PPIs. As discussed earlier in this chapter, identification and characterization of hotspots by such methods as alanine scanning mutagenesis, tethering, and SAR by NMR are slow and laborious and have not consistently resulted in the discovery of leads that are capable of further development as drug candidates. A computational approach – if validated via the discovery of actual lead compounds and drugs – has the potential to overcome the bottlenecks inherent in earlier methods, and thus would constitute a breakthrough enabling technology.

Vajda and coworkers noted that the average volume of pockets within PPI interfaces is only 54Å^3, as opposed to traditional ligand-binding sites on proteins (e.g. enzyme active sites, ligand-binding sites on

receptors) with an average volume of 260Å^3. PPI interfaces have an average of six small pockets, but it is difficult to determine which, if any, can bind a small-molecule inhibitor. Moreover, for such a pocket to bind a drug-sized inhibitor, the pocket usually must be capable of expansion. Thus a successful computational method must predict not only the ability to bind fragment-sized small molecules, but also the side-chain flexibility necessary for expansion of pockets.

Vajda's approach is known as computational solvent (CS) mapping. CS mapping is a virtual analog of such FBDD methods as SAR by NMR. In experimental fragment screening, researchers have observed that hot spots in PPI interfaces are characterized by the ability to bind a variety of fragment-sized small molecules. The number of such different small molecules, or "probes," that bind to a site predicts the potential importance of that site as a drug binding site and is predictive of the druggability of the PPI.

CS mapping is a computational method for addressing a protein molecule with a set of 16 different types of virtual small organic molecule probes, and of identifying sites on the protein that bind and cluster large numbers of probes. The method shows that virtual probes bind and cluster at only a few sites on a protein. The main hot spot on all druggable targets have been found to bind at least 16 probe clusters and, together with nearby hot spots, predicts that the site has the potential to bind drug-sized ligands.

Vajda and his colleagues used CS mapping to identify potential binding pockets as described above and combined it with a set of rules to account for the flexibility of nearby side chains, which would be necessary for the binding site to accommodate a drug-size inhibitor.

Vajda's methodology for identifying druggable sites at PPI interfaces requires only the X-ray diffraction structures of unbound proteins. An increasing number of protein structures have been determined and published over the years, and they are publically accessible via databases such as the Protein Data Bank (PDB) (http://www.pdb.org/pdb/home/home.do). Thus, in most cases, structures of proteins of interest are readily available to researchers without having to determine them *de novo*.

Vajda and coworkers validated their method by applying it to six well-studied PPI targets (Kozakov et al., 2011). (One of these targets, IL-2/IL-2Ra, has been discussed in this chapter, and others for which drugs have been discovered will be discussed in later chapters.) By "well-studied," the researchers meant targets

that had been studied via such methods as X-ray diffraction and/or NMR structure determination, site-directed mutagenesis/alanine scanning, SAR by NMR, tethering, or other types of FBLD/FBDD, or for which small-molecule inhibitors had been discovered by other means. These targets are listed in Table 2. As shown in this Table, the Vajda group's CS-mapping technology predicted the druggability or non-druggability of all these well-studied PPI targets. Moreover, CS-mapping located the same site (i.e., hotspot or collection of adjacent hotspots) on each PPI target that was capable of serving as a binding site for a small-molecule inhibitor as did experimental methods such as alanine scanning and FBLD.

Table 2: Well-studied PPI targets used to validate Vajda's CS-mapping technology

Protein	PPI partner	Small-molecule inhibitor(s) discovered?	Druggable hotspot identified via CS-mapping?
IL-2	IL-2Ra	Yes (by Sunesis)	Yes
Bcl-XL	BAK	Yes (by Abbott) †	Yes
MDM2	p53	Yes (by Roche) †	Yes
HPV-11 E2	HPV-11 E1	Yes (by Boehringer Ingelheim) *	Yes
ZipA	FtsZ	No	No
TNF-α††	TNFR1	No	No

Abbreviations: Bcl-XL = B-cell lymphoma-extra large (antiapoptotic protein of the Bcl-2 family); BAK = Bcl-2 homologous antagonist killer (proapoptotic protein of the Bcl-2 family); MDM2 = mouse double minute protein 2, a negative regulator of the p53 tumor suppressor protein; HPV-11 E2 = human papillomavirus (HPV)-11 E2 (a viral transcriptional regulator that facilitates the binding of E1 to the viral origin of replication); HPV-11 E1 = human papillomavirus (HPV)-11 E1 (HPV viral helicase; unwinds viral DNA so that it can be replicated by host enzymes); ZipA and FtsZ = two proteins that work together to mediate bacterial cell division (ZipA is homologous to actin, and FtsZ to tubulin. The ZipA/FtsZ interaction had been thought to be a potential target for antibacterial agents); TNF-α = tumor necrosis factor-alpha; TNFR1 = tumor necrosis factor receptor 1.

Notes: * Boehringer-Ingelheim suspended R&D on these inhibitors, because of the success of HPV vaccines.

† Discussed later in this report.

†† Large-molecule TNF-α inhibitors are a successful class of drugs – e.g. infliximab (Janssen Biotech/Merck's Remicade), adalimumab (Abbott's Humira), etanercept (Amgen/Pfizer's Enbrel). Although no small-molecule inhibitors of the TNF-α/TNFR1 PPI have been discovered via FBDD, Ensemble Therapeutics discovered macrocycles that inhibit this interaction, as discussed later in this report.

Source: Haberman Associates, based on Kozakov et al., 2011 **INFORMA**

Having validated their CS-mapping technology, the Vajda group then went on to apply it to a set of nine PPI targets of interest that had not been as well studied by non-virtual methods (Kozakov et al., 2011). The researchers discussed one of these targets, the eIF4E/eIF4G eukaryotic translation initiation complex (eIF4E stands for “eukaryotic translation initiation factor 4E”). eIF4E is frequently overexpressed in human cancers, and inhibition of its interaction with eIF4G (or targeting of eIF4E with an antisense compound) inhibits numerous cancer-related pathways, resulting in tumor apoptosis (Graff et al., 2008). In 2007, a collaborative group of academic researchers screened a commercial chemical library (the 16,000 compound Chembridge “DIVERSet” E library) and identified an inhibitor of the eIF4E/eIF4G interaction known as 4EGI-1; they also produced an analog that was also inhibitory (Moerke et al., 2007). However, these compounds only inhibit at the micromolar level; the binding constant for 4EGI-1 was 25μM. Structural studies with NMR indicate that a set of four amino acid residues of eIF4E interact with one of these inhibitors, 4EGI-1. However, there is no experimentally determined structure of 4EGI-1 bound to eIF4E.

The Vajda group applied its CS-mapping technology to map both unbound eIF4E and eIF4E bound to the inhibitor 4EGI-1 (Kozakov et al., 2011). Mapping of the bound and unbound protein showed that the main hotspots CS1 through CS4 form an elongated site that could support interactions with small molecules. Using a docking algorithm to place 4EGI-1 on the eIF4E protein suggested that the bound 4EGI-1 occupied only CS1 and part of CS3. This agrees with the published NMR data, which indicates that the inhibitor interacts with a set of amino acid residues found within CS1 and CS3.

Vajda and his colleagues then went on to screen another library of approximately 218,000 small organic compounds for inhibition of the eIF4E/eIF4G PPI. Among several hits, they identified a new compound, 4E1RCat (PubChem ID code 16195554) (Kozakov et al., 2011; Cencic et al., 2011). This compound inhibited the interaction between eIF4E and eIF4G with an IC_{50} of 4μM. Use of the same docking algorithm as for 4EGI-1 indicated that 4E1RCat occupies CS1 and part of CS3, but unlike 4EGI-1 also reaches into CS4. The researchers speculate that this might account for the slightly higher affinity of 4E1RCat as compared with 4EGI-1. Vajda and his colleagues consider 4E1RCat to be a lead compound for further optimization, both with respect to potency and selectivity (Cencic et al., 2011). Among other strategies, they will be using the modeling results to design new compounds that extend 4E1RCat into an adjacent binding groove.

In the new CS mapping study (Kozakov et al., 2011), Vajda and coworkers concluded that hot spots (as determined by CS mapping/side-chain flexibility and/or by previous experimental methods) are characterized by a concave shape combined with a mosaic-like pattern of hydrophobic and polar amino acid side-chains. Small-molecule ligands that bind to these sites are drug-like compounds with a hydrophobic scaffold that also possess some polar functionality. These hotspot characteristics account for their identification via CS mapping (i.e. by binding of large numbers of probe clusters) and also for the experimental finding that, in SAR by NMR studies, fragment hit rate is predictive for the probability of finding a high-affinity drug-like ligand.

Vajda's studies indicate that druggable sites on PPIs can be identified from the structure of the unbound protein, using these researchers' virtual CS mapping technology. Researchers can therefore readily identify druggable PPIs without expending efforts on laborious experimental studies and also avoiding fruitless studies on intractable PPI targets. As shown in the case of eIF4E/eIF4G, researchers can also use data from CS mapping/side chain flexibility to discover or design novel small-molecule PPI inhibitors. Such a strategy might constitute a powerful enabling technology that can help move the field of small-molecule PPI inhibitors up the technology curve, and enable the discovery and development of numerous drugs that target PPIs.

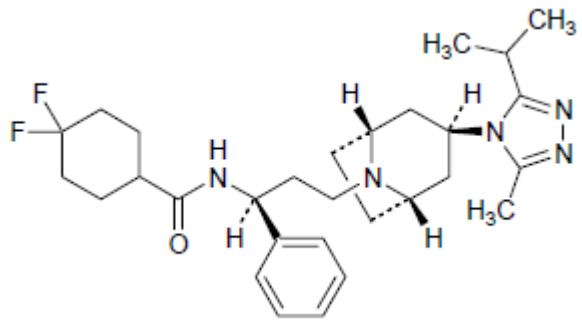
Forma Therapeutics believes that CS mapping indeed constitutes such an enabling technology. Forma's strategy is discussed later in this chapter.

Discovery of allosteric modulators of PPIs

An indirect strategy for discovering small-molecule PPI modulators involves discovering allosteric modulators. An allosteric site is a region of a protein that lies outside a binding site for the protein's natural ligand but when modulated changes the conformation of the protein in such a way that it affects the activity of the binding site. In the case of PPIs, discovering small molecules that bind to allosteric sites that resemble conventional binding sites for drugs (e.g. as found on receptors or enzymes) may be easier than discovery of direct PPI modulators. The same may apply to discovering allosteric modulators of other types of intractable targets, such as protein phosphatases.

In most, if not all cases, allosteric modulators of PPIs have been discovered serendipitously, usually using high-throughput screening (HTS). For example, the anti-HIV/AIDS drug maraviroc (Pfizer's Selzentry/Celsentri; Figure 3), a compound that was approved both in the US and in Europe in 2007, is an allosteric PPI inhibitor that was discovered via HTS (Dorr et al., 2005). It is an allosteric antagonist of the CCR5 chemokine receptor. CC chemokine receptors such as CCR5 are receptors for CC chemokines, which are so named because they contain two adjacent cysteines near their amino terminus. CCR5 is specific for the chemokine RANTES (Regulated on Activation, Normal T Expressed and Secreted, also known as CCL5.) Chemokines are a family of small cytokines, which induce chemotaxis.

Figure 3: Chemical structure of maraviroc



Source: WHO Drug Information, Vol. 18, No. 2, 2004 **INFORMA**

In addition to being bound and activated by RANTES, CCR5 is also a coreceptor (together with CD4) for entry of the most common strain of HIV into T cells. Thus maraviroc interferes with the entry of HIV, and is therefore known as an HIV entry inhibitor.

Chemokine receptors are a member of the G-protein coupled receptor (GPCR) superfamily. GPCRs are seven-transmembrane (7TM) domain receptors (i.e. integral membrane proteins that have seven membrane-spanning domains). Most of the GPCRs that are studied by pharmaceutical industry researchers bind to natural small-molecule ligands, and researchers can readily discover small-molecule drugs that competitively inhibit these ligands and thus serve as inhibitors. Such molecules represent the largest class of drugs produced by the industry, accounting for approximately half of all drugs. Chemokine receptors, however, bind to small proteins, specifically the chemokines. Their physiological activity thus involves formation of PPIs.

HIV entry also involves binding of a protein, the viral envelope glycoprotein gp120, sequentially to CD4 and CCR5.

Developing small-molecule chemokine antagonists thus has all the difficulties of developing PPI inhibitors, and only one, the allosteric inhibitor maraviroc, has reached the market. Maraviroc, and the general issue of discovering small-molecule chemokine antagonists, is discussed in more detail in Chapter 3.

The discovery of maraviroc represents part of a wave of discovery of allosteric modulators of GPCRs, mainly including modulators of GPCRs with small-molecule natural ligands, but also other chemokine receptor inhibitors (Conn et al., 2009). The GPCR allosteric modulator cinacalcet (Amgen's Sensipar/Mimpara), modulates the calcium-sensing receptor (CasR), thus increasing its sensitivity to circulating calcium. It is used in the treatment of diseases that involve certain dysfunctions in calcium homeostasis, such as hyperparathyroidism and hypercalcemia in patients with parathyroid carcinoma.

Many other allosteric modulators of GPCRs have been discovered in recent years but have not yet reached the market. Researchers hypothesize that, because GPCRs exert their signaling activities via complex ligand-mediated conformational changes, they may be a particularly "allosteric" class of proteins. Different natural ligands and drugs that bind to "orthosteric" sites on GPCRs (i.e. the sites that bind natural ligands) induce unique GPCR conformational states that activate a discrete subset of signaling pathways and cellular behaviors. This phenomenon is known as "stimulus-trafficking." Since allosteric modulators of GPCRs also work by causing conformational changes in the structures of these proteins, some of them may also give rise to functional selectivity in the actions of orthosteric natural ligands that co-bind to the GPCR. In some cases, this effect may have positive therapeutic effects. Researchers who are developing allosteric modulators of GPCRs are investigating these phenomena.

Another example of a serendipitous discovery of small-molecule allosteric modulators of PPIs is the discovery of compounds that act at an allosteric site to inhibit the subunits of the heterodimeric transcription factor core binding factor (CBF) CBFβ and Runx1 (also known as CBFα) (Gorczynski et al., 2007). The CBFB gene, which codes for CBFβ, is the target for a common chromosomal translocation, which is found in 12–15% of acute myeloid leukemia (AML) cases. The translocation produces a fusion protein between an N-terminal region of CBFβ and the coiled-coil region of smooth muscle myosin protein; this fusion protein is

known as CBFβ-SMMHC. The fusion protein exhibits anomalously tight binding to Runx1, a dysfunction that causes the leukemic phenotype of this class of AML. Since the PPI between CBFb-SMMHC and Runx1 is required for this dysfunction, the PPI represents an excellent disease target, provided it can be drugged.

The academic research group that carried out the study (led by John H. Bushweller at the University of Virginia) with the CBF system first solved the molecular structure of CBFβ and then mapped the binding site with Runx1 using alanine mutagenesis and an NMR spectroscopy method called chemical shift perturbation. They then used a virtual screening method, followed by screening via chemical shift perturbation, and finally optimization of the resulting compounds via standard medicinal chemistry methods. On the basis of their methodology, the researchers expected to discover compounds that directly modulated the PPI. However, they showed via NMR spectroscopy that the compounds bound to a novel allosteric site on CBFβ. Thus, even though these compounds were not discovered via HTS, they represent an example of serendipitous discovery of allosteric modulators of a PPI.

Unlike the PPI itself, this allosteric site was amenable in terms of shape and functionality to bind small molecules. The researchers therefore were able to discover (via virtual screening and conventional experimental screening) three additional classes of small-molecule inhibitors that bound to the allosteric site and blocked the interaction between CBFβ and Runx1. The compounds were biologically active, causing decreased proliferation of a leukemic cell line via inducing differentiation to more mature cell types and apoptosis.

The goal of the researchers was to discover, based on their initial drug discovery efforts, compounds that inhibited the leukemogenic CBFβ-SMMHC, but not CBF in normal cells. As of 2010, the researchers claimed to have preliminary evidence that shows that they have discovered compounds that inhibit the fusion protein, with minimal impact on normal cells (Estabrook, 2010). The University of Virginia Patent Foundation has filed patent applications on these compounds and is seeking licensees.

Apart from serendipitous discovery of allosteric modulators of PPIs, some researchers have been developing methods for identification of allosteric sites in proteins and discovering drugs that modulate the activity of these proteins by binding to these allosteric sites. When he was at Sunesis, James Wells and his colleagues

investigated the use of tethering for this purpose, and Wells has continued this research at UCSF. Such methods are in principle applicable to discovering allosteric modulators of PPIs.

In 2005, Sunesis researchers published a report in which they identified a cysteine-containing allosteric site on the enzyme protein tyrosine phosphatase-1B (PTP1B) (Hansen et al., 2005; Wells was not an author of this paper.) PTP1B is a potential target in type 2 diabetes and obesity. Although it is an enzyme and does not work via PPIs, its active site (and that of other protein phosphatases) is considered undruggable. The fluorogenic reagent 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABDF) forms fluorescent derivatives with thiols, including thiol groups of cysteine in proteins. ABDF was found to react selectively with a single cysteine in PTP1B, cysteine 121, leaving other cysteines in the protein unmodified. When ABDF reacts with cysteine 121, it attenuates the phosphatase activity of the enzyme, and the kinetics of this inhibition (i.e. reduction in V_{max} without affecting K_m) is suggestive of allosteric inhibition. Cysteine 121 lies outside the catalytic site of PTP1B, as expected for an allosteric site. However, it makes contact with other residues that have been shown to be important for the activity of the enzyme. This particular cysteine is highly conserved among phosphatases, and ABDF also inhibited two other protein phosphatases that were tested. Since the allosteric site contains a cysteine, tethering can in principle be used to discover drug-like compounds that specifically bind noncovalently to the allosteric site of PTB1B and of at least some other protein phosphatases.

Wells has continued to study allosteric modulation of protein activity, including the use of tethering to identify allosteric sites and to design compounds to modulate them. However, he has mainly been focusing on allosteric modulation of caspases (a class of proteases involved in apoptosis) rather than PPIs.

Design of improved chemical libraries for targeting PPIs

As we discussed in Chapter 1, commercial small-molecule compound libraries developed via combinatorial chemistry, and compound libraries of large pharmaceutical companies, generally have been poor sources of hits and leads for targeting PPIs. Researchers believe that the problem with these libraries is that they represent only a small fraction of chemical space (i.e. the “space” spanned by the total number of possible small organic molecules) and that they are biased toward compounds that modulate targets of existing

drugs. As we cited in Chapter 1, studies indicate that 83% of small natural product scaffolds and 20% of small metabolite scaffolds are not represented in commercially available libraries. This provides a measure of the poor coverage of chemical space in these libraries, which affects the use of these libraries not only for the discovery of PPI modulators but also for modulators of other types of targets as well.

Nevertheless, in a few cases (which we shall discuss in the case studies in Chapters 3–6), it has been possible to screen commercial small-molecule compound libraries, or libraries of natural products, via HTS and obtain hits that could be optimized into drug candidates (Pagliaro et al., 2004). However, in most cases, the screening of commercial small-molecule compound libraries to obtain hits and lead compounds that modulate PPIs has been fruitless.

As a result, many PPI researchers have been focusing on FBDD, as discussed earlier in this chapter. (FBDD has also been applied to small-molecule drug discovery for other types of targets, with some success.) However, other researchers focus on the area of “diversity-oriented synthesis” (DOS).

In DOS, researchers attempt to build chemical libraries that cover larger portions of chemical space than do libraries derived from standard combinatorial chemistry and that include complex “natural product-like” compounds that are likely to have biological activity. DOS researchers develop synthetic schemes that attempt to maximize the number of structures and scaffolds they can produce in as few steps as possible, to fill the largest amount of chemical space. The field of DOS has been pioneered by Stuart Schreiber (Howard Hughes Medical Institute, Broad Institute of Harvard & MIT, and Harvard University, Department of Chemistry & Chemical Biology) (Nielsen and Schreiber, 2008; Schreiber, 2009).

Diversity-oriented synthesis versus fragment-based drug design

In February 2011, Nature published a “Forum” on drug discovery, in the form of a “debate” or “point/counterpoint” on the merits of FBDD versus DOS (Hajduk et al., 2011). Representing FBDD was Philip J Hajduk of Abbott Laboratories, who had worked with Stephen Fesik on SAR by NMR and its use to discover PPI modulators. Representing DOS were two academics, Warren RJD Galloway and David R Spring of the University of Cambridge (Cambridge, UK).

To summarize the debate from the point of view of Hajduk, FBDD researchers can represent an enormous amount of chemical diversity space with a library of only a few thousand fragments. Even a modest fragment library of 1,000 fragment-sized molecules can arguably represent the diversity found in a library of tens of millions of drug-sized compounds. Moreover, compounds designed via FBDD are custom-designed to modulate a specific validated biological target. In contrast, DOS libraries are designed in a speculative manner, with the expectation that a diverse library of complex small molecules will provide hits and eventually leads for a large variety of potential biological targets. This requires a large up-front investment, mainly in compounds that will never lead to drugs that can modulate a validated biological target.

Finally, Hajduk asserts that the large, complex molecules that predominate in DOS libraries, although some of them may modulate such targets as PPIs, tend to be unlikely to have the physicochemical properties to succeed as drugs in human clinical trials. These are properties that are captured in Lipinski's Rule of Five and determine the solubility and permeability of a small-molecule compound. In contrast, compounds created via FBDD are more likely to be soluble and of low molecular weight. As a result, it may be easier to modify them to optimize their physicochemical properties.

The putative inability of compounds produced via DOS to serve as drugs may be consistent with the fact that the vast majority of DOS research is done in academia, not industry. DOS compounds that inhibit key biological targets such as PPIs involved in signal-transduction pathways are ideal "chemical tools," or "probes." Such compounds are very useful in exploring biological pathways *in vitro* (for example, in cultured mammalian cells) or in small easily permeated organisms such as the nematode *Caenorhabditis elegans*, *Drosophila* fruit fly embryos, and zebrafish embryos. The use of chemical probes for this purpose, for example screening for compounds that result in specific phenotypic changes in cells or small molecules, and then identifying the targets that are hit by these compounds, is known as chemical genetics.

However, tool compounds need not have the appropriate physicochemical properties to serve as drugs, or even to be readily optimizable into drug candidates. Chemical genetics is usually the province of academia (including Schreiber's laboratory), while discovery of drug leads is usually the province of industry. Nevertheless, as discussed later in this section, industry is becoming more interested in the fruits of DOS.

Arguing for the advantages of DOS over FBDD, Galloway and Spring assert that DOS is the best way to efficiently create drug-size, structurally diverse molecules that have otherwise escaped the attention of industry, and perhaps even of nature. DOS, but not FBDD, is also applicable to cases in which researchers wish to develop a drug for a specific disease, but do not know the specific targets that are involved in causation of the disease. Screening with a library of structurally diverse compounds could be used to identify the targets. This is the chemical genetics approach discussed above. In contrast, fragment libraries do not bind to biological targets with high potency, and cannot be used directly in chemical-genetic target identification and validation.

In general, FBDD requires that researchers not only know what the protein target is, but have a great deal of structural and even functional information about it. In most cases, this means a water-soluble protein for which an X-ray diffraction and/or an NMR structure are available. In the case of PPIs, identification of hotspots and if possible determination of their druggability, by methods such as discussed earlier in this chapter, is highly desirable.

Galloway and Spring also argue that, although fragment libraries can cover more chemical space with fewer compounds than DOS libraries, they often have limited structural diversity and tend to be biased toward compounds that satisfy the requirements of traditional medicinal chemistry, or toward aromatic compounds, which are easily detected via NMR screening (because methods such as SAR by NMR are often used in FBDD). Moreover, leads and drug candidates discovered via FBDD tend to be flat molecules. In contrast, nature, and especially protein binding sites such as PPI hotspots are three-dimensional. DOS typically results in novel, three-dimensional molecular scaffolds.

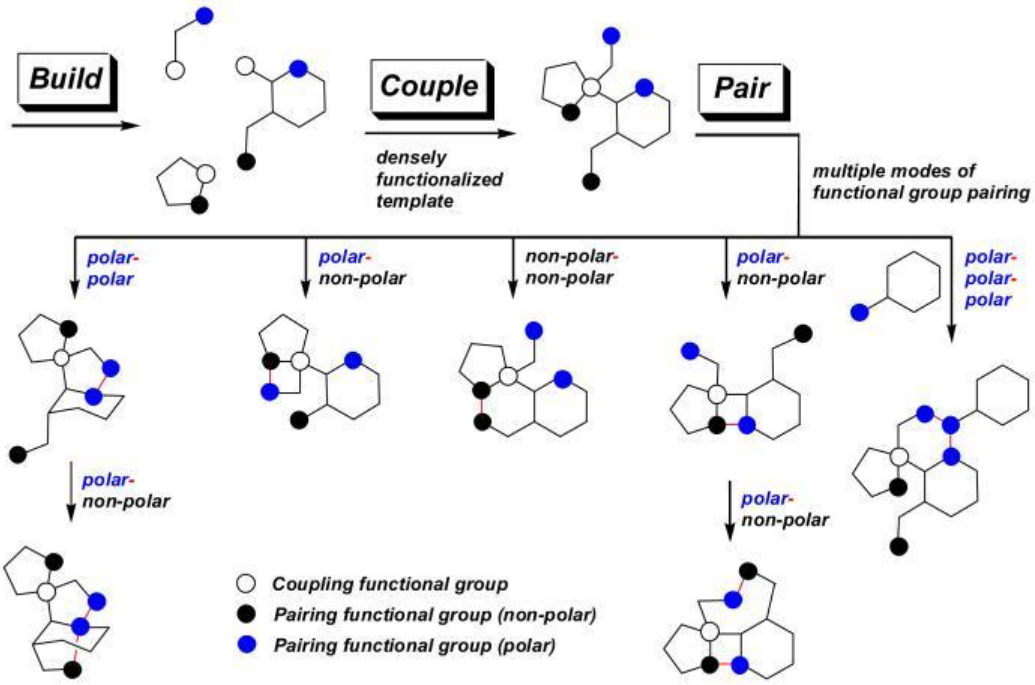
The “Build, Couple, Pair” strategy for diversity-oriented synthesis

In recent years Schreiber and his colleagues have developed a strategy for the synthesis of diverse libraries in as small a number of steps as possible (Nielsen and Schreiber, 2008; Schreiber, 2009). This modular strategy, known as “Build/Couple/Pair” (B/C/P) has been adopted by numerous other organic chemists, including Galloway and Spring and their colleagues (Galloway et al., 2010).

The three steps of the strategy, as outlined by Nielsen and Schreiber (2008) are as follows (and see Figure 4):

- **Build** – Synthesize a set of chiral building blocks containing a functional group suitable for subsequent coupling and pairing steps.
- **Couple** – The building blocks are coupled together in various combinations, controlling the outcomes in terms of stereochemistry.
- **Pair** – Intramolecular coupling that joins pairs of functional groups within molecules synthesized in the "couple" phase. This results in a library of structurally diverse, chiral compounds, which often contain rings.

Figure 4: Build, couple, pair strategy



Source: Nielsen and Schreiber, 2008 INFORMA

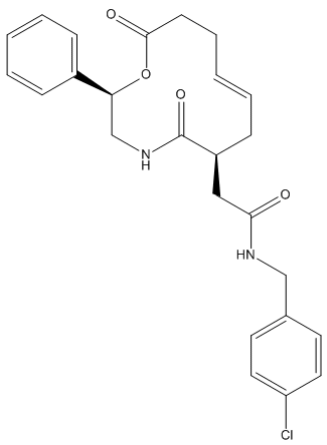
For example, in one study, researchers produced 80 compounds with diverse skeletons, using just a few steps, via a B/P/C strategy (Morton et al., 2009).

The real test of DOS synthesis strategies is whether one can screen the resulting libraries and find compounds that have biological activity, especially compounds that can modulate difficult targets such as PPIs. Such compounds have indeed been discovered via screening of DOS libraries; they modulate such

difficult types of targets as PPIs, transcription factor interactions with DNA, and multidrug resistance in pathogens (Galloway et al., 2010).

In particular, robotnikinin, discovered by the Schreiber group, binds to the extracellular protein Sonic Hedgehog (Shh) and inhibits its interaction with the 12-pass transmembrane receptor Patched 1 (Ptc1) (Stanton et al., 2009). The Shh/Ptc1 interaction activates a signal transduction pathway known as the Hedgehog pathway, which is necessary for embryonic development. This pathway has also been implicated in various cancers. The structure of robotnikinin is shown in Figure 5. Although Harvard has applied for a patent on this compound, and is seeking licensees (Harvard University, Office of Technology Development, 2011), it is likely to be useful as a probe, not a drug, unless medicinal chemists can modify it to produce a derivative with drug-like physicochemical properties. Nevertheless, as discussed in the next section, many macrocycles (robotnikinin is itself a macrocycle) have drug-like physicochemical properties and some are approved drugs, despite violating the Rule of Five.

Figure 5: Chemical structure of robotnikinin



Source: Stanton et al., 2009

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The new focus on macrocycles in academia and industry

A macrocycle is an organic compound containing a ring architecture of 12 or more atoms. Many natural products, including numerous drugs, are macrocycles. Macrocyclic compounds are also frequently made in DOS schemes such as those based on the B/P/C strategy.

In recent years, because of the rise of DOS and especially of the B/P/C strategy, macrocycles have become of great interest to synthetic organic chemists in academia. (Indeed, with the emphasis on total synthesis of natural products, macrocycles have been of interest to academic organic chemists for decades. DOS, with the prospect of easier synthesis of types of natural product-like macrocycles different from actual natural products, has simply created a new interest.) They have also recently become of interest to industry, because of the prospect of using these compounds as drugs to modulate intractable targets such as PPIs.

A leader in the industrial macrocycle field is the venture capital-funded company Ensemble Therapeutics (Cambridge, MA). In 2008, Ensemble researchers published a review on the exploration of macrocycles for drug discovery, which highlights the new interest in this class of compounds (Driggers et al., 2008).

Macrocyclic natural products have provided many drugs to the pharmaceutical industry and to medicine, including macrolide antibiotics (e.g. erythromycin and azithromycin), other antibiotics (rifampin and vancomycin), immunosuppressors (e.g. cyclosporine, rapamycin, and sirolimus), and cancer chemotherapy drugs (temsirolimus, everolimus, and epothilone) (Driggers et al., 2008). Among these compounds are certain modulators of PPIs, such as paclitaxel, epothilone B, dictyostatin, and halichondrin B. These compounds modulate (stabilize or disrupt) the interaction between the α and β subunits of the tubulin heterodimer, thus disturbing microtubule dynamics and acting as antimitotics. Other PPI modulators among macrocyclic natural products include the mammalian target of rapamycin (mTOR) inhibitor rapamycin (also known as sirolimus), which forms a complex with FK-binding protein 12 (FKBP12); this complex forms a PPI with mTOR complex 1, thus inhibiting its activity. (Rapamycin/sirolimus's anticancer derivatives temsirolimus and everolimus work via the same mechanism.) Similarly, the macrocyclic natural product cyclosporine A, an immunosuppressant, forms a complex with cyclophilin A, which forms a PPI with calcineurin. This complex inhibits the action of calcineurin, which when not inhibited activates the expression of IL-2.

As discussed in Chapter 1, although natural products had been a mainstay of pharmaceutical R&D, with the rise of HTS and combinatorial chemistry they fell out of favor. This retreat from natural products included a retreat from macrocyclic natural products. Moreover, companies have similarly not been investigating synthetic macrocycles, because of the difficulty of synthesizing these compounds, and because they violate the Rule of Five, especially with regards to molecular mass. Despite violating the Rule of Five, macrocycles

can demonstrate drug-like physicochemical properties with respect to such factors as solubility and lipophilicity, and they also can display oral bioavailability and metabolic stability, and good pharmacokinetic and pharmacodynamic properties (Driggers et al., 2008).

Given the proven therapeutic potential of macrocyclic natural products, including the potential to address challenging targets such as PPIs, the lack of attention given to macrocycles in the pharmaceutical industry has been an important reason for its lack of success in addressing challenging targets and in solving such pressing problems as the need for new drugs to combat microbial antibiotic resistance. However, there have been new developments that now put macrocycles within easier reach of medicinal chemists: first, the development of strategies for the synthesis of macrocycle-rich chemical libraries, such as B/C/P and Ensemble's proprietary technology (discussed later in this section); and second, the recent development of breakthrough methods for the synthesis of organic compounds, such as olefin metathesis (Lee and Grubbs, 2001; Yu et al., 2011). Such methods can be used in schemes for the synthesis of natural product-like and macrocycle-rich libraries, such as B/C/P.

As discussed in the review by the Ensemble researchers, macrocycles may be thought of as a class of compounds that are intermediate between small molecules and biomolecules (Driggers et al., 2008). Among the macrocycles are cyclic peptides, which biochemists might classify as biomolecules. But the Ensemble researchers attempt to make the case for considering all macrocycles as small biomolecules. Like biomolecules, some macrocycles exhibit functional sub-domains. For example, tacrolimus and its analogs have a domain that binds to FKBP12, and another domain that modulates the targeting of the bound complex to calcineurin or mTOR. Other macrocycles that exhibit functional sub-domains include the antibiotic vancomycin and the antitumor macrocycle dictyostatin.

In general, macrocycles have chemical and biological properties that are favorable for their potential as drugs compared with related non-macrocyclic compounds. For example, linear peptide compounds are readily degraded by proteolytic enzymes and are thus poor drug candidates. In many cases, creating macrocyclic versions of these peptides reduces their flexibility, and renders them resistant to proteolytic enzymes, thereby improving their metabolic stability. Moreover, macrocyclization also improves the

membrane permeability of these peptides, which can lead to improved pharmacokinetics (Driggers et al., 2008).

For example, the hepatitis C virus NS3 protease inhibitor BILN 2061 (Boehringer Ingelheim) was created by linking two side chains of a linear peptide into a macrocyclic ring (Driggers et al., 2008). This resulted in a dramatic improvement in potency over the linear peptide, as well as improved metabolic stability and favorable oral pharmacokinetics in animal models and in patients. Unfortunately, the compound was discontinued due to cardiotoxicity. Nevertheless, it illustrates the potential of macrocyclization.

Moreover, macrocyclization can position functional groups for optimal binding to a site on a target protein (e.g. a catalytic site on an enzyme or a hotspot in a PPI), thus improving affinity and/or selectively over linear compounds. For example, a tetrapeptide phosphotyrosyl mimetic binds to the SH2 phosphotyrosine recognition domain of the adapter protein growth factor receptor-bound protein 2 (Grb2). Grb2 is involved in PPIs that are critical for many signal transduction pathways. For example, it links the epidermal growth factor receptor (EGFR) tyrosine kinase to the activation of Ras and the pathways that it controls, such as the mitogen activated protein kinase (MAPK) pathway. Activating mutations in several components of this pathway are involved in such cancers as melanoma and non-small cell lung cancer (NSCLC). Macrocyclization of the tetrapeptide phosphotyrosyl mimetic stabilizes the preferred conformation for binding to the SH2 domain, which increases affinity approximately 140-fold. Further modification of this macrocycle results in a compound with a Kd (dissociation constant) of 75pM. This high-potency compound shows antiproliferative activity against cells (such as cancer cell lines) that are mitogenically driven through such signaling pathways as the MAPK pathway, which are dependent on Grb2 (Driggers et al., 2008).

In another example, researchers have designed macrocyclic derivatives of the notoriously nonspecific adenosine triphosphate (ATP)-competitive kinase inhibitors staurosporine and rebeccamycin. These derivatives are selective inhibitors for such kinases as cyclin-dependent kinases, protein kinase C (PKC), and glycogen synthase kinase 3β (GSK3β), all of which are potential drug targets (Driggers et al., 2008).

Ensemble's macrocycle synthesis technology

Ensemble's technology platform is designed to build large libraries of macrocyclic compounds, which the company calls "Ensemblins." The company synthesizes its macrocycles via its proprietary DNA-programmed

chemistry (DPC) technology. DPC allows researchers to control the chemical reactions needed for synthesis of its Ensemblins.

In DPC, each reactant or product is attached to and identified by a specific DNA tag. Hybridization to form double-stranded DNA drives the reactions between these components by bringing the reactants together, resulting in selective formation of products. This occurs under mild aqueous conditions. DPC reactions are run sequentially to make complex macrocyclic drug-like molecules, and it is possible to make tens of thousands of such compounds simultaneously in the same reaction mixture, while avoiding chemical cross-reactivity.

Ring closure reactions needed to form macrocycles are difficult to perform under normal conditions, because of competition with unwanted intermolecular reactions. DPC allows researchers to perform ring closure reactions with high fidelity due to the control provided by the hybridization of DNA molecules used to tag the specific reactants.

Once libraries are synthesized, Ensemble researchers screen them for biological activity via affinity selection. The company claims that its screening technology has allowed it to rapidly screen hundreds of thousands of macrocycles against high-value targets to generate leads. As of early 2010, Ensemble's library contained over 500,000 Ensemblins, and the company claimed that it would have a library of over one million macrocycles by the end of that year, and that it was on track to produce 4.2 million macrocycles as of early in the third quarter of 2011. This represents the largest set of synthetic macrocycles assembled by the pharmaceutical industry.

As of July 2011, Ensemble had a portfolio of 52 patents, including those licensed from Harvard University and those owned outright. In that month, Ensemble received Notices of Allowance for two new patents, both licensed from Harvard and invented by Ensemble scientific founder David Liu (Harvard Department of Chemistry and Chemical Biology) and his colleagues. Both patents (US Patent Numbers 7,771,935 and 8,017,323) are involved with Ensemble's DPC platform.

Ensemble has a pipeline of drugs in development, among which are inhibitors of PPIs. In 2009, Ensemble announced that it had discovered macrocycles that block the interaction of tumor necrosis factor (TNF) with

its receptor TNFR (Drahl, 2009). Note that the TNF/TNFR interaction was found to be undruggable by CS-mapping technology (Table 2), and other researchers had been unable to discover inhibitors of this PPI. However, Vajda's technology is designed to model fragment-based drug discovery, not macrocycles binding to PPI hotspots. This suggests that, at least in some cases, macrocycles might be able to modulate PPIs that would be deemed undruggable by other methods. However, this must be verified experimentally.

The TNF/TNFR interaction is involved in numerous inflammatory diseases, such as rheumatoid arthritis (RA). So far, the only marketed drugs that address this interaction are biologic TNF inhibitors that must be injected. An orally active TNF/TNFR inhibitory macrocycle, Ensemble's E-32712, was found to be effective in a rat model of RA. In 2009, this compound was selected for further preclinical studies. Currently, Ensemble claims to have a preclinical compound in development for the treatment of inflammation, as well as compounds in lead optimization in oncology and diabetes, as well as earlier-stage compounds in development in inflammation and pain. An Ensemble oncology compound targets the Bcl-2 family, and is thus a PPI inhibitor. Other Ensemble pipeline compounds target phosphatases and proteases.

In addition to its internal pipeline, Ensemble has collaborative programs with Bristol-Myers Squibb (BMS) and Pfizer.

The alliance with BMS, initiated in 2009, involves using Ensemble's technology platform to find leads that modulate eight high-value targets. BMS has the exclusive right to products that result from this collaboration. In April 2011, Ensemble announced that it had delivered a preclinical macrocyclic compound to BMS, which modulates one of the eight targets, an undisclosed PPI. As a result, Ensemble received an undisclosed milestone payment from BMS. The macrocycle reportedly exhibited potency and selectivity against its target, as well as favorable pharmacokinetics in preclinical studies. In January 2012, Ensemble announced the extension of its alliance with BMS. Under the extension, Ensemble will receive additional research funding from BMS. In the press release, Ensemble CEO Michael Taylor said that his company had been making progress in addressing the set of PPIs that has been the focus of the BMS collaboration with its novel macrocyclic compounds.

Ensemble's alliance with Pfizer was initiated in January 2010. It involved deployment of Ensemble's technology platform against several PPIs of potential commercial value, and collaboration to discover lead

compounds against these targets. Under the agreement, Pfizer will have development and commercialization rights to the compounds.

These alliances demonstrate the interest of two big pharma companies, BMS and Pfizer, in drugs that modulate PPIs, and the willingness of these companies to invest in alliances to discover and develop macrocyclic compounds that modulate PPIs.

Ensemble's investors include Flagship Ventures, ARCH Venture Partners, CMEA Capital, Boston University, Kisco, and Harris & Harris Group.

Cellular assays in screening for drugs that modulate PPIs

As discussed earlier, a major reason why conventional HTS usually cannot be used to discover PPI modulators is that conventional chemical libraries are not likely to contain suitable compounds. The other issue in screening for PPI modulators is that HTS assays are usually designed to identify compounds that inhibit catalytic or receptor activity of proteins, not the docking of two proteins. Researchers have therefore been using cellular assays that can assess the activity of whole intracellular pathways or portions of pathways to identify compounds that inhibit these pathways via disruption of key PPIs. This section includes two examples of such cellular screening assays.

Ligand's STATs technology

One approach (which has been successful in identifying PPI modulators that will be discussed in Chapter 3) is the proprietary "STATs technology," which was developed by Ligand Pharmaceuticals (La Jolla, CA) (Tian et al., 1998). Many cytokines and growth factors work by binding to the extracellular domains of their receptors and dimerizing them, resulting in the activation of an intracellular signal transduction pathway. The dimerization of some receptors (for example, the granulocyte colony-stimulating factor (G-CSF) receptor) triggers intracellular signaling through a pathway that results in the phosphorylation of members of a class of proteins known as signal transducers and activators of transcription (STATs). Upon phosphorylation, STATs form homodimers and translocate into the nucleus. The STAT homodimers bind to specific DNA sequences in STAT-responsive promoters, which drive the transcription of specific sets of genes. Ligand researchers developed a high-throughput fluorescent reporter assay in which a reporter gene is driven by a synthetic

STAT-responsive promoter that has been stably transfected into a G-CSF responsive cell line. As will be discussed in Chapter 3, Ligand's successful collaboration with GlaxoSmithKline (GSK) to discover and develop small-molecule thrombopoietin (TPO) receptor agonists was made possible by the development of Ligand's STATs technology, and Ligand has continued to use this technology more recently to discover additional early-stage PPI modulators.

BioImage's Redistribution technology

BioImage (Søborg, Denmark) developed a proprietary cellular screening assay technology called "Redistribution" (Almholt et al., 2004). In 2006, Thermo Fisher Scientific (Waltham, MA) acquired BioImage. Thermo now markets BioImage Redistribution cellular assays.

Redistribution technology focuses on pathways that involve intracellular translocation of a signaling protein from one intracellular compartment to another, such as from the cytoplasm to the nucleus or vice versa. Many signal transduction pathways involve activation of a key cytoplasmic protein (for example by phosphorylation), followed by translocation into the nucleus, or stabilization of a signaling protein in the cytoplasm, which is then free to enter into the nucleus. Once in the nucleus, these proteins may activate transcription of specific sets of genes. The activation or stabilization of these key cytoplasmic proteins is usually dependent on one or more protein-protein interaction events, such as the formation of a protein complex necessary for phosphorylation of the key protein.

Redistribution technology involves measuring the translocation of a fluorescently labeled signaling protein of interest in living cells. These assays can then be used to screen for compounds that alter the translocation of the signaling protein. Thermo considers Redistribution assays to be examples of high-content screening (HCS) assays (Thermo Scientific 2011a). HCS, which is based on cellular assays, microscopy, and fluorescent read-outs, involves a combination of cell biology, molecular tools such as fluorescent labeling of specific proteins, automated high resolution microscopy, and robotic handling. It also involves informatics to handle and analyze the large amounts of data that are generated by these assays.

HCS enables researchers to study the activity of drugs and their targets in a cellular context, which more closely resembles *in vivo* physiology than in the case of HTS using traditional biochemical assays. Compounds to be tested are inherently screened for membrane permeability as well as activity against a

target in the context of the cellular environment. Moreover, HCS, depending on the design of the assay, allows researchers to screen compounds for effects on whole pathways, which is usually not possible in biochemical-based HTS. Redistribution assays are an example of whole-pathway screening.

Furthermore, HCS allows researchers to design assays that allow them to obtain multiple readouts from the same assay, for example by using differently colored fluorescent reporters. This can save on screening costs. In the case of Redistribution assays, even if only a single fluorescent readout is obtained, the assays also provide information about changes in cell morphology or motility, as well as cellular toxicity, due to treatment with specific compounds. Toxic compounds and those with poor cellular permeability are thus screened out early in the drug discovery process.

Since Redistribution assays are whole-pathway assays, any hits and leads discovered via these assays may be compounds that modulate known types of single druggable targets such as kinases or proteases, or they may be compounds that modulate more than one such target. Alternatively, they may be modulators of PPIs, especially in signaling pathways that are critically dependent on such interactions.

An example of a Redistribution assay marketed by Thermo is the β-catenin Redistribution assay (Thermo Scientific, 2011b). This assay gives researchers a readout of the Wnt pathway, in terms of the localization of β-catenin in the nucleus, which is the critical step in this pathway. Dysregulation of the Wnt pathway is a causative factor in several types of cancer, especially in colorectal cancer, and also multiple myeloma (MM), hepatocellular carcinoma (HCC), and B-cell chronic lymphocytic leukemia (B-CLL).

Soluble extracellular factors that are members of the Wnt family activate the canonical Wnt pathway via an extracellular receptor complex (van Noort and Clevers, 2002). Engagement of the extracellular receptor complex by Wnt family ligands leads to disruption of the “destruction complex.” This cytoplasmic multiprotein complex includes as one of its components the protein adenomatous polyposis coli (APC). When Wnt receptors are not engaged by their ligands, the “destruction complex” remains intact. Then another component of the complex, glycogen synthase kinase 3 (GSK3), phosphorylates β-catenin. β-Catenin is a multifunctional protein that is involved both in signal transduction and in intercellular adhesion. Phosphorylation targets β-catenin for degradation in the cytoplasm; this is the reason why the APC complex is called the “destruction complex.”

When Wnt family ligand binding leads to the disruption of the destruction complex, β-catenin accumulates in the cytoplasm and moves into the nucleus. There it binds to a transcription factor, and activates transcription of a set of downstream genes, including the oncogene Myc and other genes that mediate cell proliferation.

In precancerous colonic adenomas and in the colorectal cancers that they can evolve into, APC is usually mutated, thus disrupting formation of the destruction complex. This results in the constitutive stabilization and nuclear localization of β-catenin, and constitutive activation of the specific transcription factor and its downstream genes. In other types of cancer that involve constitutive Wnt pathway activation, β-catenin also becomes stabilized via other means. We shall discuss compounds that inhibit a PPI involved in Wnt pathway transcription in Chapter 4.

The Thermo/BioImage Redistribution assay is based on a genetically engineered cell line that stably expresses human β-catenin fused to a C-terminal moiety of enhanced green fluorescent protein (EGFP). The assay enables researchers to assay for antagonists of β-catenin nuclear translation or, alternatively, for agonists of that translation. In one type of antagonist assay, the cells are first treated with an inhibitor of GSK3. This inhibitor is itself an agonist of β-catenin nuclear localization, and thus cells treated with it will have nuclei that contain the fluorescent protein. Researchers can then screen for compounds that block nuclear translocation of β-catenin in cells treated with the GSK3 inhibitor.

In addition to its whole-pathway Redistribution assays, BioImage developed a Redistribution assay that directly measures the interaction between p53 and its physiological inhibitor, the human analog of mouse double minute 2 (MDM2) protein. This human analog is known as HDM2. Thermo now markets this assay (Thermo Scientific, 2011c). (The p53-HDM2 PPI, and the discovery of small-molecule inhibitors of this PPI, such as nutlin-3, which is mentioned later in this section, will be discussed in Chapter 5.)

This Redistribution assay is based on the unique translocation property of the human cyclic adenosine monophosphate (cAMP) phosphodiesterase PDE4A4, which can localize to compact cytoplasmic foci. HDM2 is fused to PDE4A4B, and p53, which forms a PPI with HDM2, and is labeled with green fluorescent protein (GFP). Treatment with the PDE4A4 Redistribution agonist RS25344 induces localization of PDE4A4 into cytoplasmic foci. In this assay, since PDE4E4 is fused to HDM2 bound to p53, the fluorescent label on p53 also localizes to the cytoplasmic foci.

When cells carrying this construct are treated with an inhibitor of the HDM2/p53 PPI, such as nutlin-3, GFP-labeled p53 is liberated from the cytoplasmic complex, and moves into the nucleus, which as a result will carry a fluorescent label. This assay can thus be used to screen for inhibitors of the HDM2/p53 PPI via HCS. In principle, researchers can use this technology, known as GRIP (green fluorescent protein-assisted readout for interacting proteins) technology, to design HTS assays for inhibitors of other PPIs.

Forma Therapeutics: moving small-molecule PPI modulator discovery up the technology development curve

As discussed earlier in this chapter, the discovery of compounds to modulate PPIs appears to be a "premature technology," which awaits the development of sets of enabling technologies to move it up the technology development curve, and thus enable researchers to readily discover and develop such compounds. Forma Therapeutics (Watertown, MA – the company also has locations in Branford, CT and Singapore) has a technology strategy that, among other things, is designed to do this. Forma's focus is oncology, especially the discovery and development of drugs that address "challenging targets" that have eluded the efforts of drug discovery researchers. The company's interests include PPIs, as well as tumor metabolism, epigenetics, autophagy, necroptosis (i.e. programmed necrosis), and the ubiquitin system. (Some of the targets in the other areas of Forma's interest are or may be PPIs; in most cases, the nature of Forma's targets is closely guarded.)

In addition to its major focus on cancer, Forma has a collaboration with Cubist Pharmaceuticals to discover novel antibacterials. This program is based on Forma's chemistry platform, and any compounds discovered under this agreement will be developed by Cubist.

Forma was founded in May 2007, launched by the Novartis Option Fund. Steven Tregay, who had been a managing director of Novartis Option Fund, became Forma's CEO. As of April 2008, the Novartis Option Fund had invested $4m in Forma. In January 2009, Forma announced in a press release its "debut as an integrated, global drug discovery company." (Forma Therapeutics, 2009). According to the press release, Forma was founded by leading researchers from the Broad Institute of Harvard and MIT. These included Stuart Schreiber (discussed earlier in this chapter), Todd Golub (Director of the Broad's Cancer Program,

and an investigator at the Dana-Farber Cancer Institute and Howard Hughes Medical Institute), and Michael Foley (Director of the Broad's Chemical Biology Platform). Other members of Forma's founding team mentioned in the press release were Steven Tregay, and Nikolai Kley (now Forma's Vice President of Discovery Biology, and formerly Vice President and Head of Research, GPC Biotech). Since then, the company has added additional R&D leaders, including two with Novartis backgrounds – Kenneth Bair (now Forma's Senior Vice President and Head of Research and Development, and earlier in his career Unit Head of Oncology at Novartis), and Jaime Escobedo (now Vice President, Biology, and formerly Head of Translational Biology and Pharmacology at Novartis Oncology).

Forma's planned focus was to be the discovery and development of drugs to address "essential oncology targets that have been elusive to the best scientists in the industry." As of January 2009, Forma had received funding of $25m from Novartis Option Fund and Bio*One Capital of Singapore, among other sources. In December 2009 Forma announced that it has closed on a Series B financing that raised a total of $25.5m. The new round of financing was led by new investor Lilly Ventures, with participation from existing investors Novartis Option Fund and Bio*One Capital of Singapore. Forma collaborator Cubist Pharmaceuticals also joined as a new equity investor. In addition to its venture capital deals, Forma derives revenues from collaborations with Cubist, Novartis, and The Leukemia & Lymphoma Society.

The focus of Forma's technology platform and R&D strategy is on chemical biology. The core of Forma's platform was spun out from technology developed by its Broad Institute founders, in DOS and chemical synthesis, cell-based screening, genomic and proteomic profiling, and target identification. And in October 2008, as discussed earlier, Forma acquired SolMap Pharmaceuticals, thus acquiring the CS mapping technology developed by Vajda and his colleagues for identifying PPI hotspots and for discovering novel small-molecule PPI inhibitors.

Forma's small-molecule compound library, which includes DOS libraries, contains over 500,000 compounds. As discussed earlier, DOS enables researchers to produce natural product-like compounds (including macrocycles), and to fill in areas of chemical space not addressed by conventional combinatorial chemistry, thus producing libraries with a higher probability of including hits for PPI targets. Forma combines screening of these libraries with large-scale X-ray crystallography, CS mapping, and other structure-based drug design

technologies. The company also possesses a proprietary high-speed chemical synthesis platform. These technologies enable Forma researchers to rapidly create new chemical scaffolds and to produce and optimize lead compounds in a matter of weeks. Forma combines its chemistry technologies with proprietary cell-based screening methods to identify compounds that modulate desired pathways and to gain an understanding of how these compounds may behave in living cells.

The components of Forma's technology platform are:

- chemical library largely based on diversity-oriented synthesis, coupled with a proprietary high-speed chemical synthesis platform
- structure-based drug design technologies, including large-scale X-ray crystallography
- computational solvent mapping technology
- cell-based screening.

As can be seen from this list, Forma's platform contains most of the advanced technologies applicable to discovery of small-molecule PPI modulators discussed earlier in this chapter.

PPIs have been an intended major focus of Forma from its founding. In the January 2009 press release, Reinhard Ambros, Head of the Novartis Venture Funds, stated that he believed that Forma's integrated drug discovery platform would position the company "for success in areas that are considered 'holy grails' of oncology drug discovery such as interfering with protein-protein interactions or functional activity of transcription factors." (Forma Therapeutics, 2009)

Forma's entire corporate strategy – and especially its technology strategy – runs contrary to the prevailing trends of the 2009–12 period. This has been a period of retrenchment in Big Pharma R&D, and biotechs have been following lean or even virtual technology strategies to conserve cash. Biotechs have also been focusing on one or a few pipeline products, with the usual goal of being acquired by big pharma. In contrast, Forma has been building a large R&D operation and aiming at building a large and diverse pipeline. According to Forma's CEO Steve Tregay, as of June 2011 Forma had been working on drug discovery/development programs against 10 novel cancer metabolism targets, and was also working to

identify compounds that modulate PPIs (Timmerman, 2011). However, Forma has released few details on its drug discovery and development programs.

The company also appears to be aiming at remaining an independent company. For example, in June 2011, Forma entered into an agreement with Genentech (which is a member of the Roche group) in which the latter company paid to acquire the worldwide rights to a single preclinical-stage cancer metabolism program from Forma, which if the drug reaches the market could enable Forma to generate returns for its investors without going public or being acquired, and which would allow Forma to continue developing other drugs as an independent company (Timmerman, 2011).

Under the terms of the agreement, Forma received an upfront payment and research support, and Genentech agreed to cover all development costs for the drug, and to make milestone payments. If the drug is approved by the FDA, Genentech has the option to acquire the full rights to the drug. Instead of paying royalties, Genentech would make an asset buyout payment to Forma's investors, plus further milestone payments to Forma if certain sales goals are met.

The ability of Forma to contract such an agreement is driven not only by the strength of its technology platform and R&D effort, but by the great interest in targeting cancer metabolism for the development of novel oncology drugs by big pharma and biotech companies.

Forma has entered into several collaborations that focus on the discovery and development of PPI modulators. In January 2009, shortly after its formal "debut," Forma entered into a collaboration with Novartis, the company that had the biggest role in launching Forma. The agreement was worth a potential $200m in upfront and milestone payments, as well as royalties on sales of any products developed through the collaboration. The focus of the collaboration was to use Forma's technology platform to develop inhibitors for an undisclosed protein-protein interaction target with relevance to cancer. The agreement includes an upfront fee and potential milestones totaling over $200m as well as royalties. In July 2009, Forma entered into another collaboration (financial terms undisclosed) with Novartis to use its cell-based screening platform to discover inhibitors for undisclosed cancer-relevant PPIs.

In March 2010, Forma entered into an agreement with the Leukemia & Lymphoma Society (LLS) (White Plains, NY) to discover and develop inhibitors of the PPI between the transcription repressor Bcl-6 and its corepressor silencing mediator of retinoid and thyroid hormone receptors (SMRT). This interaction is key to signaling pathways that are involved in diffuse large B-cell lymphoma, a type of aggressive non-Hodgkin's lymphoma. Another effort to develop BCL6/SMRT inhibitors is discussed in Chapter 4.

This collaboration between Forma and LLS builds on the original agreement between the two entities, which was signed in July 2009. The original agreement was to focus on ten promising small-molecule compounds that had been discovered by LLS grant-funded academic researchers. Forma was to use its CS mapping technology, together with its screening and medicinal chemistry technologies to optimize and prioritize molecules that LLS and its partners might advance into clinical trials. LLS said that it had committed substantial, multi-year funding to support both collaborations with Forma as part of its Therapy Acceleration Program (TAP). TAP is designed to advance into the clinic, in partnership with biotech companies, therapies with good prospects of providing near-term benefits to leukemia and lymphoma patients.

In January 2012, Forma announced that it had entered into a collaboration with Boehringer Ingelheim to discover and develop novel oncology compounds. The collaboration will focus on discovering small-molecule modulators of PPIs involved in cancer-relevant disease pathways. Under the agreement, Forma will receive a total of $65m in up-front payments and research funding to screen for and optimize compounds against multiple targets over the next four years. Forma may also receive up to $750m in pre-commercial milestone payments. Further financial details were not disclosed. However, Forma CEO Steven Tregay said that the agreement will offer Forma shareholders several opportunities to realize early returns from assets developed under the new agreement. As discussed earlier, a key financial goal of the Genentech agreement was to enable Forma investors to realize returns without an acquisition or an IPO.

In summary, Forma has built a technology platform that is designed to move the discovery of small-molecule modulators of PPIs up the technology curve; the company's platform is also applicable to other areas of drug discovery, including the discovery of drugs that modulate other types of challenging targets. The company also has a strong drug discovery program in cancer metabolism, which is of great interest to the biotech/pharma industry today. Forma has been able to attract early-stage venture capital investment and

early-stage big pharma alliances in a difficult market for any early-stage biotech investment. It thus becomes a leading company in the advancement of the PPI modulator field.

Conclusions

As discussed in this chapter, so far the discovery of small-molecule modulators of PPIs has been on a “one compound at a time” basis. The discovery of such compounds has been made possible by the development of sets of tools based on such methods as X-ray diffraction, alanine scanning mutagenesis, and fragment-based drug discovery. Central to the development of this set of tools has been a small group of pioneering researchers and pioneering companies. Leading companies in this field have especially included Genentech, Roche, Sunesis, and Abbott. Leading researchers in this field have especially included Timothy P. Clackson (formerly at Genentech), James Wells, Michelle Arkin, and Daniel Erlanson (all formerly at Sunesis), and Stephen Fesik (formerly with Abbott). (Although some of these researchers were not mentioned in the text of this chapter to this point, all are mentioned as authors in the References to this chapter.) Academic labs have also played an important role in these studies, and continue to do so.

However, as the result of the difficulty in making PPI modulator development a commercial proposition on a consistent basis, all these companies abandoned or cut back on their PPI modulator programs, and several of these researchers have moved on to academic positions. An exception is Daniel Erlanson, who founded Carmot Therapeutics (San Francisco, CA) in 2008. Carmot obtained an exclusive license to Sunesis’s proprietary fragment-based lead discovery (FBLD) technology, including the tethering methodology, in February 2010. Carmot focuses on using this technology to discover compounds such as kinase inhibitors and PPI inhibitors, all of which are in the discovery stage. With respect to PPIs, Carmot is working (in collaboration with Boston University, and funded via a Small Business Innovation Research (SBIR) grant) to discover compounds that modulate the NF-κB signaling pathway, which is involved in inflammation and cancer. The company is specifically interested in discovering inhibitors of the NEMO/IKK (NF-κB essential modulator/IkB kinase) PPI, a key component of the pathway.

Chapters 3–6 of this report consists of collections of case studies, each one of which describes the discovery and in some cases the development of PPI modulators via the sporadic “one compound at a time” approach.

Although the discovery and development of PPI modulators has been sporadic, the fact that any at all have been developed and reached the market validates the field, by showing that such development is possible.

What the small-molecule PPI modulator field has lacked has been a powerful suite of technologies and strategies to enable the regular and consistent discovery of such compounds. Thus we have called small-molecule PPI development a "premature technology." This chapter, however, describes the development of a suite of enabling technologies that may well have the capability of moving the field up the technology development curve, and making development of these compounds a commercial proposition.

Among these new technologies is CS mapping, which builds on the pioneering technologies for hotspot determination, and FBLD/FBDD developed by the researchers and companies mentioned earlier in this section. CS mapping and other structural biology and FBLD/FBDD technologies are complemented by new chemical technologies, such as DOS and Ensemble's proprietary macrocycle synthesis technology, as well as cell-based screening technologies. This new suite of technologies is represented, for example, by Forma's technology platform, as outlined in above.

As a result of the development of the new suite of enabling technologies, both venture capital investment and pharma companies have been moving back into PPI discovery. Selected large pharmaceutical companies with active programs or partnerships in small-molecule PPI modulator discovery are listed in Table 3. This table focuses on the discovery of PPI modulators rather than development of compounds from older PPI programs, to illustrate the return of the industry to early-stage PPI modulator R&D.

Table 3: Selected large pharmaceutical companies with active small-molecule PPI modulator discovery programs

Company name	Comments
Bristol-Myers Squibb	Alliance with Ensemble
Pfizer	Alliance with Ensemble
Novartis	Key player in launching Forma and has an alliance with that company; also collaborating with academic groups in the PPI field
Ajinomoto	Alliance with Interprotein Corporation (Osaka, Japan)

Source: Haberman Associates

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Most of the programs listed in Table 3 involve alliances with either Ensemble or Forma. Among the other programs, a collaboration between Novartis and an academic group will be discussed in Chapter 4. The alliance between Ajinomoto and the Japanese biotech Interprotein Corporation (Osaka, Japan) was announced in September 2010 but little information is available about it. Interprotein's technology platform is based on the work of Takao Matsuzaki in structure-based drug design (SBDD), and the company focuses on the discovery of small-molecule PPI inhibitors designed to replace mAb drugs that bind to such macromolecules as VEGF and interleukin-6. The alliance between Ajinomoto and Interprotein will initially involve a one-year drug discovery effort aimed at an undisclosed target selected by Ajinomoto. The financial terms were not disclosed.

As exemplified by the companies listed in Table 3, several large pharmaceutical companies, as well as biotech companies, have been moving back into small-molecule PPI discovery, including such leaders as Novartis, Pfizer, and BMS. This involves companies in the US, Europe, and Japan. Meanwhile, such companies as GSK (in collaboration with Ligand), Roche, and Abbott are continuing with development programs for small-molecule PPI modulators derived from older discovery programs, as we shall discuss in subsequent chapters. With the emergence of powerful enabling technologies for discovery of these compounds, and given the new strategic importance of PPIs as targets discussed in Chapter 1, we expect additional companies to enter the field.

Chapter 3 Small molecules targeting protein-protein interactions of cell-surface receptors

Summary

- Many cell-surface receptors (e.g. receptors for cytokines, chemokines, and growth factors, as well as integrins) exert their physiological functions by interacting with proteins or peptides.
- Researchers and companies have developed large-molecule drugs – including recombinant proteins and mAbs – that target many of these receptors. These include biologic inhibitors of tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF), and recombinant versions of proteins that are ligands for cell-surface receptors, such as epoetin alfa (Amgen's Epogen / Johnson & Johnson's Procrit), and granulocyte colony-stimulating factor (G-CSF) (Amgen's filgrastim/Neupogen.) These biologics are among the most successful drugs on the market today and, in the case of agents whose patents have expired, they are the targets of biosimilar development.
- Some researchers and companies would like to develop small-molecule receptor modulators to substitute for these biologics. Such small-molecule drugs might be orally available, and would have the potential to be less expensive and safer than the corresponding biologics. In some cases, developing biologic drugs to modulate such receptors has not been possible, so any small-molecule receptor modulators would be the first drugs to be developed that address these receptors.
- The development of small-molecule drugs that modulate these receptors has been difficult. Nevertheless, three such drugs have reached the market: eltrombopag (Ligand/GSK's Promacta/Revolade), maraviroc (Pfizer's Selzentry/Celsentri), and plerixafor (Genzyme's Mozobil).
- Other compounds are in development, including Ligand/GSK's GSK2285921/LGD-4665 (a follow-up compound to eltrombopag; Phase II), Ligand's early-stage EPO and G-CSF agonists, SARcode's ophthalmologic compound SAR1118 (Phase III), Ensemble's macrocyclic TNF antagonists, and numerous chemokine receptor antagonists, including ChemoCentrix/GSK's Phase III Crohn's disease agent Traficet-EN/GSK786.

Introduction

Many cell-surface receptors exert their physiological functions by interacting with proteins or peptides. These include, for example, receptors for cytokines, chemokines, and growth factors, integrins (which bind to extracellular matrix proteins or to members of the immunoglobulin superfamily such as ICAM-1 (intercellular adhesion molecule-1), Fc receptors (which bind to the Fc regions of antibodies), and many others.

As discussed in Chapter 1, researchers and companies have developed large-molecule drugs – including recombinant proteins and mAbs – that target many of these receptors. These include biologic inhibitors of TNF and VEGF, and recombinant versions of proteins that are ligands for cell-surface receptors, such as erythropoietin (epoetin alfa, Amgen's Epogen and Johnson & Johnson's Procrit, for treatment of anemia associated with dialysis to treat chronic renal failure and cancer chemotherapy) and granulocyte colony-stimulating factor (G-CSF) (Amgen's filgrastim/Neupogen, for treatment of neutropenia associated with cancer chemotherapy or bone marrow transplantation, or to increase the number of blood hematopoietic stem cells prior to collection by leukapheresis for use in hematopoietic stem cell transplantation.

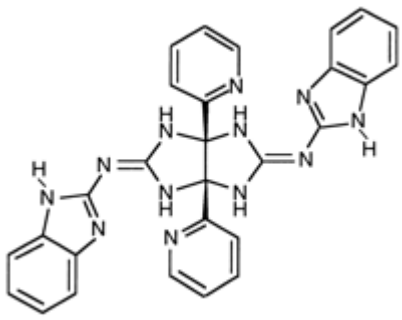
These biologics are among the most successful drugs on the market today, and in the case of agents whose patents have expired, they are the targets of biosimilar development. Nevertheless, some researchers and companies would like to develop small-molecule receptor modulators to substitute for these biologics. Such small-molecule drugs might be orally available, and would have the potential to be less expensive than the corresponding biologics, even biosimilars. They would also be expected to lack the potential for the immunologic adverse effects of large-molecule drugs. Moreover, for some receptors that will be discussed later in this chapter, developing biologic drugs has proven to be difficult or impossible. In these cases, any small-molecule receptor modulators would be the first drugs to be developed that address these receptors.

In the case of receptors for which biologic modulators are on the market, the receptors constitute very highly validated targets. Thus, for developers of small-molecule modulators, these targets have been de-risked, especially compared with novel, unprecedented targets. This would be expected to make development of small-molecule drugs that address these receptors attractive to some companies.

Small-molecule agonists of cytokine receptors

Most small-molecule PPI modulators that researchers and companies are developing or would like to develop are PPI inhibitors or antagonists. However, developers of small-molecule modulators of cytokine receptors are aiming to develop small-molecule cytokine mimetics, which are agonists. Efforts to develop such small-molecule cytokine agonists on a commercial basis began in the 1990s, with research at Ligand Pharmaceuticals. As discussed in Chapter 2, Ligand researchers developed a proprietary cellular screening assay known as the "STATs technology" (Tian et al., 1998). Ligand researchers and their collaborators from SmithKline Beecham (now GlaxoSmithKline (GSK)) used this technology to discover SB-247464, a small-molecule nonpeptide mimetic of murine G-CSF (Tian et al., 1998). G-CSF (as with many other cytokines and growth factors) binds to the extracellular domains of its receptors and dimerizes them, resulting in the activation of intracellular signaling through a pathway that results in the phosphorylation of members of the STAT class of signaling proteins. In Ligand's assay, the phosphorylated STATs form homodimers, translocate into the nucleus, and bind to a synthetic STAT-responsive promoter that drives expression of a fluorescent reporter gene. The researchers screened small organic compounds (the publication gave no details as to the nature of the chemical library or compound file that was screened) for activity with this assay, and selected SB-247464 for further study. The structure of SB-247464 is given in Figure 6.

Figure 6: Chemical structure of SB-247464



Source: Tian et al., 1998

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In the above fluorescent assay, SB-247464 had 30% of the activity of G-CSF. SB-247464 induced the same pattern of tyrosine phosphorylation of downstream signaling proteins as G-CSF. As with G-CSF, SB-247464 induced the formation of granulocytic colonies *in vitro* from mouse bone marrow, and also induced a dose-

dependent increase in neutrophil counts when injected into mice. SB-247464 induced tyrosine phosphorylation of the G-CSF receptor, but not the interleukin-3 (IL-3) receptor, consistent with being specific for the G-CSF receptor.

In later studies, the researchers found that SB-247464, like G-CSF, dimerizes the extracellular domains of the murine G-CSF receptor (Doyle et al., 2003). This dimerization is dependent on the presence of zinc ions, and SB-247464 appears to bind to extracellular domains of the G-CSF receptor in a zinc ion-dependent manner. SB-247464 has two zinc-binding (i.e. chelation) sites, which presumably are involved in zinc-dependent binding to the G-CSF receptor.

SB-247464 is a symmetrical molecule (Figure 6), and each of the symmetrical “halves” of the molecule has a zinc-binding site. This suggests that zinc-complexed SB-24764 interacts with two different extracellular domains of G-CSF, resulting in their dimerization on the cell surface. In support of this model, a related nonsymmetrical compound, SB-250017, which has only one zinc-binding site, can bind to the extracellular domain of the receptor but cannot dimerize it. SB-250017 acts as an antagonist of SB-247464, but has no effect on the activation of the receptor by G-CSF.

As show in the original study on the discovery of SB-247464 (Tian et al., 1998), this molecule has no activity on the human G-CSF receptor. This is despite the lack of mouse-human species specificity of G-CSF protein. The researchers constructed a chimeric G-CSF receptor in which the amino-terminal half of the extracellular domain that contains the G-CSF binding region was murine in origin, and the carboxy-terminal half was human. SB-24764 had no activity on this chimeric receptor. This shows that SB-24764 binds to a different site on the murine G-CSF receptor from the binding site of G-CSF.

Until recently (as discussed below), Ligand and/or GSK researchers were unable to discover a small-molecule compound that was active as an agonist of the human G-CSF receptor. So the initially promising discovery of a small-molecule G-CSF receptor agonist did not result in a corresponding human therapeutic as of the late 1980s through to the early to mid 2000s.

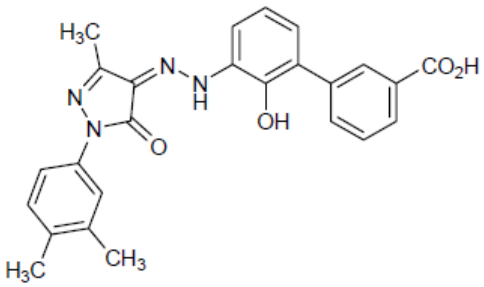
Ligand's small-molecule thrombopoietin (TPO) receptor agonists

Despite the lack of success identifying a small-molecule human G-CSF receptor agonist, the researchers used the STATs technology to discover small-molecule nonpeptide agonists of the human thrombopoietin (TPO) receptor (Erickson-Miller et al., 2005). Optimization of their initial compound, SB-394725, led to the development of eltrombopag (SB-497115-GR) (Ligand/GSK's Promacta), which was approved by the FDA in 2008 and the European Medicines Agency (as Revolade) in 2010.

The small-molecule TPO agonist eltrombopag provides an alternative to recombinant human TPO, which can elicit anti-endogenous TPO antibodies in some patients, resulting in profound thrombocytopenia (Li et al., 2001).

The structure of eltrombopag is shown in Figure 7. Eltrombopag is the only synthetic small-molecule direct (i.e. not allosteric) PPI modulator to reach the market so far. It is approved for the treatment of the rare disease idiopathic thrombocytopenic purpura (ITP). ITP is the condition of having an abnormally low platelet count (thrombocytopenia) of no known cause. Eltrombopag is also in Phase III clinical trials to treat low platelet count in patients with cirrhosis of the liver due to hepatitis C (in which low platelet counts may be a contraindication for treatment with interferon), and in Phase II trials in oncology. A follow-on compound to eltrombopag, GSK2285921 (formerly LGD-4665; developed by Ligand and licensed to GSK) is in Phase II clinical trials in oncology. With respect to oncology, a related compound to eltrombopag, SB-559457, but not recombinant human TPO, was found by academic researchers to be specifically toxic to primary human myeloid leukemia cells in culture (Kalota and Gewirtz, 2010).

Figure 7: Chemical structure of eltrombopag



Source: WHO Drug Information, Vol. 20, No. 3, 2006 **INFORMA**

Ligand's preclinical small-molecule EPO and G-CSF receptor agonists

More recently, Ligand reports that, using its STATs technology, it has discovered small-molecule agonists of the human receptors for erythropoietin (EPO) and for G-CSF (Ligand Pharmaceuticals, 2011) Apparently, Ligand/GSK's failure to discover a human G-CSF agonist earlier was due to limitations in the chemical libraries they used for screening. The STATs cellular assay technology was and is good for discovering such compounds, provided chemical libraries screened in these assays contain viable hits.

Ligand presented preclinical data on one of its lead EPO receptor agonists, LG5640, at the 52nd American Society of Hematology (ASH) Annual Meeting in December 2010.

The company has discovered a lead series of small-molecule, selective EPO receptor agonists (including LG5640) that display partial efficacy compared with recombinant human EPO in several models of EPO-induced erythropoiesis. Several compounds in this series have been identified as potential preclinical development candidates. These compounds induce erythroid maturation in human bone marrow hematopoietic cells positive for the pluripotent hematopoietic stem cell marker CD34. They also display oral bioavailability in the rat and monkey. Ligand's EPO receptor agonists are expected to lack the excessive erythropoietic stimulation of such recombinant-protein erythropoiesis-stimulating agents (ESAs) as Amgen's Epogen, Johnson & Johnson's Procrit, and Aranesp (Amgen's longer-acting form of recombinant EPO). Thus Ligand's experimental EPO receptor agonists might lack the adverse effects of ESAs, such as an increased risk of adverse cardiovascular complications in patients with kidney disease, and a potential increase in mortality in cancer patients.

In Ligand's new small-molecule G-CSF receptor agonist program, the company has identified lead compounds that stimulate G-CSF-dependent cell growth, increase STAT phosphorylation, and induce differentiation of human bone marrow cells into granulocytes. The company states that further optimization of the chemical series should provide orally available compounds to treat neutropenia with improved convenience and an improved safety profile compared with current injectable recombinant G-CSF.

Ligand is seeking licensing partners for its new small-molecule EPO and G-CSF receptor agonist programs.

Small-molecule integrin antagonists

The integrin superfamily of proteins consists of cell-surface receptors that mediate attachment between cells and either the extracellular matrix (ECM) or other cells. Among these proteins is the leukocyte integrin lymphocyte function-associated antigen 1 (LFA-1). LFA-1 is found on such leukocytes as T cells, B cells, macrophages, and neutrophils, and is involved in recruitment to sites of infection or inflammation. LFA-1 on T cells binds to the immunoglobulin superfamily member ICAM-1 (intercellular adhesion molecule-1) on, for example, antigen-presenting cells and endothelial cells. The LFA-1/ICAM-1 PPI plays an important role in such processes as T-cell activation, T-cell homing to peripheral lymphoid organs and sites of inflammation (Dustin et al., 2005; Graf et al., 2007). This PPI is thus a target for the discovery of drugs to treat inflammatory conditions.

As discussed in Chapter 2, in the 1990s and early 2000s Sunesis had been working on the discovery and development of PPI inhibitors, based on its tethering technology platform. However, after that time, the company changed its focus to development of oncology drugs that do not work via PPI inhibition.

Among Sunesis's PPI inhibitor programs was a program aimed at the discovery and development of LFA-1/ICAM-1 inhibitors. Sunesis did discover such inhibitors, via use of its tethering-based FBDD platform. These compounds are described, for example, in US Patent Number 7,314,938 (Shen et al., 2008) and in a 2010 publication by Sunesis researchers (Zhong et al., 2010). According to the latter publication, their compounds potently inhibited both human T-cell migration and T-cell activation in *in vitro* assays by disrupting the LFA-1/ICAM-1 PPI. One of their compounds showed good pharmacokinetic properties and oral availability in rodents and inhibited neutrophil migration in a murine peritonitis model.

In 2007, Sunesis licensed its then-discontinued LFA-1 inhibitor program to SARcode Corporation (now SARcode Bioscience) (Brisbane, CA). As a result of this licensing agreement, Sunesis received a $250,000 license fee and a $250,000 note convertible into preferred stock. In March 2009, Sunesis sold to SARcode all its intellectual property and know-how related to the LFA-1 inhibitors program for $2m in cash. In August 2011, SARcode repaid three promissory notes that had originally been issued to Sunesis, including the principal of $1.0m plus accrued interest.

SARcode, a venture-backed ophthalmic biotech company, was founded in 2006. Sunesis's LFA-1 antagonist program became SARcode's lead development program following the above 2007 licensing agreement.

In September 2008, SARcode initiated a Phase I clinical trial of a topically administered small-molecule LFA-1 product candidate, SAR1118, for dry-eye syndrome, which involves inadequate tear production. It is a common and often chronic condition that affects approximately 20m people in the US. Dry eye varies in severity and etiology. A major contributing factor toward the development of dry eye is inflammation caused by T-cell infiltration, proliferation, and inflammatory cytokine production. This can lead to reduction in tear film quality and ocular surface damage.

SARcode reported the results of a randomized, placebo-controlled multicenter Phase II trial of SAR1118 in dry eye in May 2011. The agent was safe and well tolerated, and demonstrated statistically significant improvements in tear production and visual function. In September 2011, SARcode reported the enrollment of the first patient in a pivotal Phase III study of SAR1118 ophthalmic solution in the treatment of dry eye syndrome.

In addition to dry eye, SARcode plans to test SAR1118 in a broad range of ocular inflammatory conditions including diabetic macular edema.

Chemokine receptor antagonists

As discussed in Chapter 2, chemokines are members of a family of small cytokines, which induce chemotaxis. Chemokines and their receptors are attractive drug targets because of their role in inflammatory diseases.

Chemokine receptors are members of the GPCR superfamily. Most GPCRs that are studied by pharmaceutical industry researchers bind to natural small-molecule ligands, and researchers have discovered and developed numerous small-molecule drugs that are competitive inhibitors of these ligands. These GPCR antagonists represent the largest class of drugs produced by the industry. Chemokine receptors, however, bind to small proteins, the chemokines. Chemokines binding to their receptors thus represent a class of PPIs. Thus discovering small-molecules that directly inhibit chemokine receptors at their chemokine binding sites has all the difficulties of discovering PPI modulators. This is despite the fact that

chemokine receptors are GPCRs, for which pharmaceutical companies have discovered numerous antagonists via standard methods of medicinal and combinatorial chemistry.

However, discovering small-molecule chemokine receptor antagonists that act via an allosteric mechanism might be expected to be easier, which is in fact the case. As discussed in Chapter 2, researchers hypothesize that because GPCRs exert their signaling activities via complex ligand-mediated conformational changes, they may be a particularly "allosteric" class of proteins. Different natural ligands and drugs that bind to "orthosteric" sites on GPCRs (i.e. the sites that bind natural ligands) induce unique GPCR conformational states that activate a discrete subset of signaling pathways and cellular behaviors. Since allosteric modulators of GPCRs also work by causing conformational changes in the structures of these proteins, some of them may also give rise to functional selectivity in the actions of orthosteric natural ligands that co-bind to the GPCR (Conn et al., 2009).

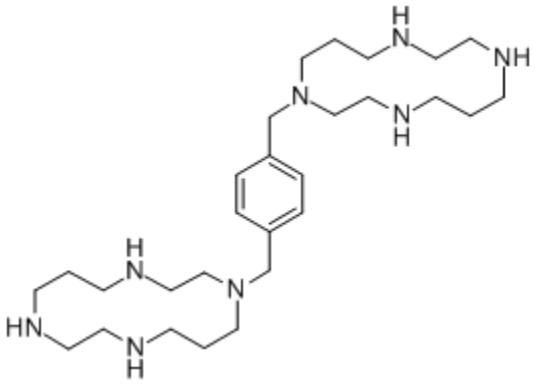
In the case of GPCRs – including chemokine receptors – whose natural ligands are peptides or proteins, allosteric sites (defined as binding sites for known allosteric modulators of these receptors) are located in distinct sites on the receptor proteins from the orthosteric peptide binding sites. Specifically, chemokines bind to orthosteric sites that are located in the extracellular domains of their receptors. Allosteric sites on chemokine receptors, however, are located in transmembrane domains that are distant from the chemokine binding sites (Conn et al., 2009). Small-molecule allosteric modulators that bind to these sites were discovered via fairly standard medicinal chemistry and high-throughput screening, sometimes augmented with structure-based drug design. Thus, although orthosteric binding sites on chemokine receptors (and on other GPCRs that have peptide ligands) have proven so far to be intractable for the discovery of small-molecule modulators, the discovery of drug-like small-molecule allosteric modulators of chemokine receptors is much more feasible.

However, the development of small-molecule chemokine receptor antagonists has not been easy, because the diseases addressed by these compounds usually have complex biology. Most of the agents that have been entered into clinical trials have failed. A recent review concludes that clinical failures may be due to poorly predictive animal models and target redundancy (Horuk, 2009). So far, only two small-molecule chemokine antagonists have entered the market. One is the allosteric CCR5 antagonist maraviroc (for

treatment of HIV/AIDS) mentioned in Chapter 2. The other is the CXCR4 inhibitor plerixafor (Genzyme's (originally AnorMed's) Mozobil).

Plerixafor (Figure 8) is a type of macrocyclic compound known as a bicyclam and was derived from an impurity in the synthesis of cyclam. This agent was thus serendipitously discovered. It was originally developed for use in the treatment of HIV infection (De Clercq et al., 1992) but was eventually repurposed for its current use (Davies et al., 2007). It is used in combination with G-CSF to mobilize hematopoietic stem cells to the peripheral blood for autologous transplantation in patients with non-Hodgkin lymphoma and multiple myeloma. Plerixafor (as a zinc complex) is a partial antagonist of the chemokine receptor CXCR4 and an allosteric agonist of CXCR7 (Kalatskaya et al., 2009). It is CXCR4's involvement in hematopoietic stem cell homing that accounts for the ability of plerixafor to mobilize these cells into the peripheral circulation. Plerixafor was approved by the FDA in December 2008, and in Europe in May 2009. It has orphan drug status in both jurisdictions.

Figure 8: Chemical structure of plerixafor



Source: www.chemicalbook.com

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Selected small-molecule chemokine modulators in development are listed in Table 4.

Table 4: Selected chemokine receptor modulators in development

Compound	Chemokine receptor	Comments
Maraviroc (Pfizer's Selzentry/Celsentri)	CCR5	Marketed; HIV entry inhibitor; approved for the treatment of HIV infection
Plerixafor (Genzyme's Mozobil; originally AnorMED)	CXCR4 (partial agonist); CXCR7 (allosteric agonist)	Marketed; used in combination with G-CSF to mobilize hematopoietic stem cells to the peripheral blood for autologous transplantation in cancer patients; macrocyclic compound; serendipitously discovered as a derivative of an impurity and originally developed for treatment of HIV infection
Traficet-EN (GSK786) (ChemoCentrix/GSK)	CCR9	Phase III, Crohn's disease
Reparixin (formerly repertaxin; Dompé)	CXCR1/CXCR2	Phase II, primary graft dysfunction after lung and kidney transplantation; discontinued. Phase II, undisclosed indication; potential utility in breast cancer
SCH 527123/navarixin (Merck; originally Schering-Plough)	CXCR1/CXCR2	Phase II, chronic obstructive pulmonary disease (COPD)
SB656933 (GlaxoSmithKline)	CXCR2	Phase II, ulcerative colitis
Cenicriviroc (formerly TBR-652) (Tobira Therapeutics)	CCR5 and CCR2	Phase IIb, HIV infection
CCX140 (ChemoCentryx)	CCR2	Phase II, diabetic nephropathy
CCX354 (ChemoCentryx)	CCR1	Phase II, rheumatoid arthritis
PF-4136309 (Pfizer)	CCR2	Phase II, hepatitis C infection with abnormal liver enzymes

Source: Haberman Associates

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ChemoCentryx (Mountain View, CA) specializes in the discovery and development of small-molecule chemokine and chemoattractant receptor inhibitors. It is developing three agents listed in Table 4. The most advanced of these, in Phase III clinical trials in Crohn's disease in partnership with GSK, is the CCR9

antagonist Traficet-EN (GSK789). ChemoCentryx is also developing two Phase II agents, the CCR2 antagonist CCX140 (for treatment of diabetic nephropathy), and the CCR1 antagonist CCX354 for treatment of rheumatoid arthritis.

The case of reparixin (formerly repertaxin), being developed by Dompé Farmaceutici (Milan, Italy), is an interesting example of potential drug repurposing (Fulmer, 2010). Reparixin targets the chemokine receptors CXCR1 and CXCR2, both of which are receptors for interleukin-8 (IL-8), a well-studied proinflammatory chemokine that is a major mediator of inflammation. Reparixin had been in Phase II development for the prevention of primary graft dysfunction after lung and kidney transplantation, but it failed in clinical trials. Dompé has also intended to test the agent in another, undisclosed indication.

Meanwhile, researchers at the University of Michigan and the Institut National de la Santé et de la Recherche Médicale (INSERM) in France used gene expression profiling to identify a breast cancer stem cell signature. These academic researchers found that the IL-8 receptor CXCR1 was among the genes almost exclusively expressed in breast cancer stem cells compared with its expression in the bulk tumor (Fulmer, 2010).

Researchers led by Max Wicha (University of Michigan Comprehensive Cancer Center, Ann Arber, MI) who is a cofounder of anti-cancer stem cell therapeutic developer OncoMed Pharmaceuticals (Redwood City, CA), carried out studies aimed at targeting CXCR1 in breast cancer stem cells (Ginestier et al., 2010). The researchers found that both reparixin and an anti-CXCR1 antibody reduced the number of breast cancer stem cells in a human breast cancer cell line *in vitro*. And after four days, both CXCR1 antagonists abolished the entire cell population, even though stem cells were only around 2% of the population. The researchers showed that both CXCR1 antagonists induced the production of soluble Fas ligand (FASL), an apoptotic mediator. The FASL bound to its receptor on bulk tumor cells, which triggered cell death via apoptosis.

Administration of either reparixin or reparixin plus docetaxel reduced tumor growth in mice carrying primary human breast cancer xenografts, compared with a saline control. The combination treatment was more effective than either agent alone. Other experiments in mice showed that reparixin could reduce breast cancer metastasis (Ginestier et al., 2010).

As of 2010, Dompé was interested in finding new indications for repertaxin. The company was aware of Wicha's research and was interested in collaborating with his group to repurpose repertaxin for cancer. (Fullmer, 2010). According to a 2011 disclosure of Wicha's financial relationships, he reported receiving research funding from Dompé (Chustecka, 2011).

Small-molecule antagonists of the TNF/TNFR PPI

As discussed in Chapter 2, Ensemble Therapeutics (Cambridge, MA), a company that utilizes its macrocycle-based technology platform to discover and develop compounds that modulate PPIs and other challenging targets, announced in 2009 that it had discovered macrocycles that block the interaction of tumor necrosis factor (TNF) with its receptor TNFR (Drahl, 2009). As discussed in Chapter 2, this is despite the fact that the TNF/TNFR PPI has previously proven to be particularly intractable by other researchers.

The TNF/TNFR PPI is involved in numerous inflammatory diseases such as rheumatoid arthritis (RA), and TNF is the target of several large-selling biologic TNF inhibitors. As discussed in Chapter 2, Ensemble selected an orally active TNF/TNFR inhibitory macrocycle that it discovered, E-32712, as the basis of its inflammatory disease program, and the company currently claims to have a preclinical compound in development for this indication.

Conclusions

Using the sets of technologies that were developed by the pioneers of small-molecule PPI modulator discovery in the 1980s to mid-2000s (see Chapter 2), as well as combinatorial chemistry, medicinal chemistry, and HTS, researchers have discovered modulators of several types of cell surface receptors that interact with protein or peptide ligands. Among them is the only direct PPI modulator not based on a natural product to reach the market, the thrombopoietin (TPO) receptor agonist eltrombopag (Ligand/GSK's Promacta/Revolade).

Also among these modulators of cell-surface receptors are numerous chemokine receptor inhibitors, the most advanced of which are listed in Table 4. Among these compounds are the marketed chemokine receptor antagonists, maraviroc (Pfizer's Selzentry/Celsentri) and Plerixafor (Genzyme's Mozobil). As

discussed earlier, many of these chemokine antagonists have been shown to be allosteric receptor modulators, and at least the majority of the rest are likely to be allosteric effectors as well.

Meanwhile, Ensemble Therapeutics has been developing early-stage TNF receptor antagonists, one of which is in preclinical testing. This was based on Ensemble's novel, proprietary synthetic macrocyclic compound technology platform, which was discussed in Chapter 2.

The modulators of cell surface receptors that interact with proteins or peptides that were discussed in this chapter other than chemokine receptors are listed in Table 5.

Table 5: PPI modulators in development targeting cell-surface receptors that interact with non-chemokine proteins or peptides

Receptor	Compound	Comments
Thrombopoietin (TPO) receptor	Eltrombopag (SB-497115-GR) (Ligand/GSK's Promacta/Revolade)	Agonist; approved for idiopathic thrombocytopenic purpura (ITP); in Phase III trials in hepatitis C
Thrombopoietin (TPO) receptor	GSK2285921 (formerly LGD-4665); Ligand/GSK	Agonist; Phase II, oncology
Erythropoietin (EPO) receptor	LG5640 and other lead agonist compounds; Ligand	Agonist; advanced discovery stage; might lack the adverse effects of current biologic agents
Granulocyte colony stimulating factor (G-CSF)	Lead optimization; Ligand	Agonist; should have improved convenience and might have an improved safety profile compared with current biologic agents
Lymphocyte function-associated antigen (LFA-1)/ intercellular adhesion molecule-1 (ICAM-1) PPI	SARcode's SAR1118 (originally developed by Sunesis)	Antagonist; Phase III, dry-eye syndrome; LFA-1/ICAM-1 is a target in inflammatory conditions, including dry eye.
Tumor necrosis factor(TNF)/TNF receptor (TNFR) PPI	Ensemble's E-32712 and other lead macrocyclic compounds	Antagonist; preclinical, inflammatory diseases; should have improved convenience over current biologic agents

Source: Haberman Associates

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As with other classes of PPI modulators, the application of some of the recently developed technologies discussed in Chapter 2 should make faster and more effective the discovery of compounds that modulate cell-surface receptors interacting with protein or peptide ligands. For example, although Ligand's cell-based STATs screening assays constitute a proven technology for the discovery of agonists of the receptors of many growth factors and immune system modulators, its success appears to have been limited by the use of chemical libraries that give inadequate coverage of chemical space. The use of libraries based on DOS or Ensemble's macrocycle technology would be expected to increase the success of the STATs technology in discovering small-molecule receptor agonists.

In several cases (e.g. Ligand and GSK's growth factor receptor agonist programs and Ensemble's TNF antagonist programs), small-molecule compounds developed in such programs might be expected to compete with or replace current biologic drugs. This would result in new-generation products that would be more convenient (i.e. orally-available) and which might have an improved safety profile over the biologic agents. Moreover, the small-molecule compounds would have the potential to be less expensive than the biologics, which must be manufactured by expensive cell culture-based methods. Several of these biologics have gone off patent, and European regulatory authorities have approved several biosimilar versions of epoetin alfa and filgrastim (G-CSF). The US FDA has recently gained the authority to approve biosimilars, but none has yet been approved. Although biosimilars are designed to be less expensive than the corresponding biologics originally developed by innovator companies, they are only marginally less expensive. That is mainly because they still must be made using expensive cell culture-based methods. Moreover, it is difficult to make exact copies or equivalent versions of most biologics, so there is always the question as to whether a biosimilar will work as well in any given patient as the original version. Small-molecule receptor modulators, if proven in clinical trials, may be a better (and potentially less expensive) solution.

Moreover, there has been a growing trend for big pharma, which has been traditionally focused on small-molecule drugs, to expand into biologics, often via mergers and acquisitions. Biologics (and especially mAbs) are becoming the industry's largest-selling drugs (Hirschler, 2009). Most of these agents target cell-surface receptors or, in the case of TNF, their ligands. If researchers could develop consistent methods to produce small-molecule ligands of many of the receptors of these biologics, this would have the potential to change a

major strategy of the industry. However, whether researchers can accomplish that remains unproven, and the timeline for any such change would be around a decade away.

Chapter 4 Small molecules targeting intracellular signaling pathways

Summary

- Signal transduction and intracellular signaling pathways are fundamental to cell and organism physiology, in health and disease. They often become dysregulated in cancer, metabolic diseases, immune diseases, and other major diseases.
- Although drug developers have successfully targeted tractable signaling receptors with small-molecule drugs and with biologics for many years, intracellular signaling pathways have until relatively recently been inaccessible to drug discovery researchers. The discovery and development of kinase inhibitors, which began in the late 1990s/early 2000s, represents a very significant breakthrough.
- Many intracellular signal transduction pathways that are dysregulated in cancer and other diseases remain inaccessible to drug discovery researchers, since they contain few if any “druggable” targets, and are driven by key components that have so far been intractable. PPIs are critical components of all signaling pathways, and researchers would like to find ways to address the “undruggable” PPIs that are central to these pathways.
- Academic researchers at Harvard Medical School, in collaboration with Novartis, have discovered several small-molecule inhibitors of the Tcf/β-catenin PPI, a transcription factor complex that is central to the Wnt pathway. This pathway is dysregulated in subsets of several types of cancer, especially colorectal cancer, multiple myeloma (MM), hepatocellular carcinoma (HCC), and B-cell chronic lymphocytic leukemia (B-CLL). This research involved structural and mutagenesis studies of the PPI (which identified a hot spot), followed by assay development and screening of natural product libraries. Novartis researchers have concluded that the compounds identified warrant further studies to determine the feasibility of testing them in human clinical trials.
- Academic researchers led by Ari Melnick (Cornell University Medical College) have discovered a small-molecule inhibitor of the BCL6/SMRT PPI in diffuse large B-cell lymphoma (DLBCL). The researchers

performed structural and mutagenesis studies (which identified a hot spot), performed computer-aided drug design, and used their models for virtual screening of 1,000,000 commercially available compounds. Compound selection was based on chemical diversity, drug-likeness, immediate commercial availability, and ability to block BCL6-mediated transcriptional repression in a cellular assay. The researchers identified a lead compound, which specifically killed BCL6-positive lymphoma cell lines and BCL6-positive tumor cells in xenograft models. The researchers are optimizing their lead compound to develop a clinical candidate for BCL6 targeted therapy for DLBCL.

- Researchers at the Leibniz Institute for Molecular Pharmacology have discovered small-molecule A-kinase anchoring protein (AKAP)-protein kinase A interaction disruptors for the potential treatment of chronic heart failure. AKAPs are scaffolding proteins that tether protein kinase A (PKA) and other signaling proteins to specific intracellular sites. The tethering of PKA (also known as cAMP-dependent protein kinase) via a PPI with an AKAP results in the compartmentalization of cAMP signaling within the cell. The AKAP18δ isoform serves as a scaffold for organizing the adrenaline-beta-adrenoreceptor-cAMP-PKA signaling pathway in cardiac muscle cells. The researchers developed a screening assay for disruption of the AKAP18δ-PKA PPI, and screened a library of over 20,000 “drug-like” compounds. They identified nine compounds, and selected one of them, FMP-API-1, for further studies. FMP-API-1 disrupted the AKAP18δ-PKA PPI with a micromolar dissociation constant, and worked via an allosteric mechanism. Higher affinity drug-like small-molecule AKAP-PKA PPI antagonists are still sought.
- As illustrated by the three case studies in this chapter, researchers have shown that it is possible to target PPIs involved in intracellular signaling pathways with small-molecule agents, producing “tool compounds” that enable these signaling pathways to be probed. In some cases it has been possible to achieve sufficient optimization to identify compounds that can be taken into the clinic.

Introduction

Signal transduction is a process by which extracellular signals mediate changes within a cell via intracellular signaling pathways. Typically, signal transduction begins when an extracellular signaling molecule activates its receptor, which can be a cell-surface receptor or an intracellular receptor such as a nuclear receptor. Upon activation, receptors mediate changes in intracellular target molecules, which initiate cascades of

molecular changes that propagate through pathways. The end result is a physiological response, for example cellular differentiation, growth and/or proliferation, secretion of signaling molecules such as growth factors or cytokines, cellular motility or adhesion, or apoptosis. Signal transduction and intracellular signaling pathways are fundamental to cell and organism physiology, in health and disease.

Signal transduction pathways often become dysregulated in cancer, metabolic diseases, immune diseases, and other major diseases. Therefore, academic researchers and drug discovery researchers in industry have been keenly interested in targeting these pathways. Researchers and companies have traditionally targeted tractable signaling receptors such as GPCRs that have small-molecule ligands, and nuclear receptors such as steroid receptors. More recently, they have developed biologics that target growth factor and cytokine receptors, which have generally not been amenable to small-molecule drug discovery. Some of the leading biologics in this class, as well as novel strategies to address their targets with small molecules, were discussed in Chapter 3.

However, intracellular signaling pathways have until relatively recently been inaccessible to drug discovery researchers. The discovery and development of kinase inhibitors, which target kinase enzymes that are key mediators of intracellular signaling, represents a very significant breakthrough. This began with the discovery and development of imatinib (Novartis's Gleevec/Glivec) in the late 1990s / early 2000s and has continued with the discovery and development of an ever increasing number of kinase inhibitors. Most of these compounds are used in the targeted treatment of various types of cancer, often involving a "personalized medicine" approach.

Despite the breakthrough development of kinase inhibitors, many intracellular signal transduction pathways that are dysregulated in cancer and other diseases remain inaccessible to drug discovery researchers. For example, as mentioned in Chapter 2, phosphatases, which are key components of important signal transduction pathways, are considered "undruggable."

Most importantly, the "undruggable" PPIs are key components of all signaling pathways, especially PPIs between transcription factors, and PPIs that form multicomponent protein complexes that are key mediators of intracellular signaling. Examples of both were discussed in Chapter 2, such as the multicomponent "destruction complex" that is involved in the destruction or movement into the nucleus of β-catenin in the Wnt

pathway, and the multicomponent transcription factor known as core binding factor (CBF), which is deregulated in a subset of AML.

The only marketed PPI modulators that target signal transduction pathways are a few natural product-derived compounds. These are the mTOR inhibitor rapamycin/sirolimus and its derivatives temsirolimus and everolimus, and the calcineurin inhibitory immunosuppressant cyclosporine A. These were also discussed in Chapter 2. Otherwise, PPI modulators that target signal transduction pathways have yet to be developed. When many, if not most researchers in the field think of "small-molecule modulators of PPIs," they mean small-molecule PPI modulators that target signal transduction pathways. This illustrates the potential importance of this area, even though most medicinal chemists and others believe that developing such compounds will be difficult or impossible.

This chapter discusses several case studies involving developing small-molecule PPI modulators that target signal transduction pathways.

Small-molecule inhibitors of the oncogenic Tcf/β-catenin transcription factor complex

In Chapter 2, we discussed a cellular assay to assess the localization of β-catenin in the nucleus, which is the critical step in the Wnt pathway. Dysregulation of the Wnt pathway is a causative factor in several types of cancer, especially in colorectal cancer, and also in subsets of multiple myeloma (MM), hepatocellular carcinoma (HCC), and B-cell chronic lymphocytic leukemia (B-CLL). Despite the central role of the Wnt pathway in these cancers, the development of therapies has been hampered by the limited number of druggable targets in the Wnt pathway. Researchers have therefore been looking for alternative means of targeting the Wnt pathway, including targeting the PPIs that are critically involved in the pathway.

Central to the Wnt pathway is the "destruction complex," a multicomponent cytoplasmic protein complex that includes (among others) the proteins adenomatous polyposis coli (APC) and glycogen synthase kinase 3 (GSK-3). When the "destruction complex" is intact, GSK-3 phosphorylates β-catenin, which is a multifunctional protein that is involved both in signal transduction and in intercellular adhesion. This phosphorylation targets β-catenin for degradation in the cytoplasm. When the destruction complex is

disrupted (which in normal physiology occurs via signaling from Wnt family ligands bound to their cell-surface receptor), β-catenin accumulates in the cytoplasm, and moves into the nucleus. There it binds to transcription factors of the T-cell factor (Tcf) family, including Tcf4, the major Tcf expressed in stem cells of the gut and in colorectal cancer. In the absence of β-catenin, Tcf proteins are transcriptional repressors. β-Catenin binding changes Tcf proteins from repressors into transcriptional activators that activate a set of downstream genes, including the oncogene c-Myc and the cell-cycle protein cyclin D1.

In the case of precancerous colonic adenomas or the colorectal cancers that they may evolve into, APC is usually mutated; thus no destruction complex can form. This results in constitutive stabilization of β-catenin, which can freely move into the nucleus and bind to Tcf4. In the case of other cancers caused by dysregulation of the Wnt pathway, β-catenin also becomes stabilized, via other genetic changes that do not involve ACP.

In 2004, a group led by Ramesh Shivdasani (Harvard Medical School, Dana-Farber Cancer Institute, and Brigham and Women's Hospital, Boston MA), including researchers from the Novartis Institutes for BioMedical Research (Cambridge, MA), discovered several small-molecule inhibitors of the human Tcf/β-catenin PPI (Lepourcelet et al., 2004).

Shivdasani's group (Poy et al., 2001), as well as others (Graham et al., 2000), had previously determined crystal structures of Tcf/β-catenin complexes; these studies were complemented by an alanine scanning mutagenesis study by another research group (Fasolini et al., 2003). As with most PPIs, the interface between the two proteins occurs over a large surface area. However, the structural and mutagenesis studies identified a hot spot in this interface. This is a small hydrophobic pocket that is critical for binding and may accommodate a small-molecule inhibitor.

The above structural studies showed that the interaction between Tcf and β-catenin requires a minimal amino-terminal Tcf binding fragment and the central domain of 12 armadillo repeats in β-catenin (amino acids 134–668; armadillo repeats are named after the *Drosophila* Armadillo protein, which is a β-catenin homolog.) The researchers designed an enzyme-linked immunosorbent assay (ELISA) involving release of an alkaline phosphatase-tagged Tcf binding fragment from its complex with a β-catenin fragment absorbed

to an ELISA plate. They used this assay to screen approximately 7,000 purified natural products from proprietary (i.e. Novartis's) and public collections (Lepourcelet et al., 2004).

Eight compounds were found that gave reproducible, concentration-dependent release of the Tcf fragment at less than 10 micromolar concentration. The structures and purity of these compounds (most of which are complex, multi-ringed planar compounds with multiple hydroxyl groups) were then determined. The sources of these compounds include fungi, actinomycetes, and a marine sponge.

The researchers performed several confirmatory *in vitro* biochemical assays for disruption of the Tcf/b-catenin PPI and for specificity (i.e. little or no disruption of other PPIs and protein-DNA interactions). They then tested compounds that were active in the biochemical assays for activity in cellular assays, including in cells containing fluorescent reporter genes that are under the transcriptional control of Tcf, and by testing for inhibition of transcription of downstream genes, including those coding for c-Myc and cyclin D1, and for the specificity of inhibition of these downstream genes, and not related genes such as that coding for cyclin E. The researchers then tested the compounds *in vivo* in *Xenopus* embryos for inhibition of β-catenin-mediated axis duplication and for specific inhibition of colon cancer cell proliferation. Of the tested compounds, two fungal derivatives – PKF115-584 and CGP049090 – gave the best results in all the assays. It is these two compounds that were tested in preclinical studies.

Despite the apparent specificity of disruption of the Tcf/β-catenin PPI by these two compounds, the molecular mechanisms by which the compounds act on this PPI are not clear. At the time of publication of their 2004 report (Lepourcelet et al., 2004), the researchers were working on mapping the docking sites of the compounds in β-catenin and/or Tcf, but they have not reported on these studies since. Since these compounds also interfere with APC/β-catenin complexes, their action may be mediated through β-catenin. Moreover, the structure of PKF115-584 resembles, but is not identical to, the structure of calphostin C, which is a protein kinase C (PKC) inhibitor. Nevertheless, the researchers' biochemical and *Xenopus* studies show that the target of PKF115-584 is the Tcf/β-catenin interface. It is possible that PKF 115–584 may also inhibit PKC; conversely it may be possible that calphostin C's inhibition of Wnt signaling may be due to disruption of the Tcf/b-catenin PPI.

Preclinical studies of PKF115-584 and CGP049090

In a study published in 2007 (Sukhdeo et al., 2007), researchers at the Dana-Farber and at Brigham and Women's Hospital tested PKF115-584 in human MM cells *in vitro* and in a xenograft model. The compound blocked β-catenin/TCF-regulated transcription (CRT) as shown by a reporter assay, blocked expression of the Wnt target genes c-MYC and Cyclin D1, activated apoptosis genes and induced cytotoxicity in MM cells *in vitro*, and inhibited tumor growth and prolonged survival in the xenograft model.

In a study published in 2010, researchers from Novartis and their academic collaborators in Europe tested PKF115-584 and CGP049090 in B-CLL *in vitro* and in a xenograft model. They also targeted Lef-1, the member of the Tcf/Lef family that is overexpressed in B-CLL and that has the same function as Tcf in the Wnt pathway, with a specific small interfering RNA (siRNA). The researchers found that targeting Lef-1 with the siRNA resulted in induction of apoptosis in primary B-CLL cells and inhibited proliferation of the JVM-3 cell line (a B-CLL model cell line transformed by Epstein-Barr virus) *in vitro*. PKF115-584 and CGP049090 efficiently killed primary B-CLL cells *in vitro*, while normal B cells were not significantly affected. Killing of B-CLL cells by these compounds was via apoptosis. In a JMV-3 xenograft model, administration of PKF115-584 or CGP049090 resulted in cessation of tumor growth and improved survival. The two compounds appeared to be well tolerated and resulted in no toxicity-related death. The researchers concluded that targeting Lef-1 is a new and selective therapeutic strategy for B-CLL. They further concluded that CGP049090 and/or PKF115-584 deserve further preclinical and clinical evaluation in B-CLL and other Wnt pathway-dependent cancers.

Compounds identified as inhibitors of the Tcf/β-catenin PPI have also been tested in two other cancers. In a study in HCC at the Asian Liver Center at Stanford University School of Medicine, PKF115-584, CGP049090, and another of the Shivdasani group's compounds, PKF118-310, were found to induce cytotoxicity in human HCC cell lines *in vitro*, and to suppress tumor growth and induce apoptosis in tumor cells in a human HCC xenograft model (Wei et al., 2010).

In a study in AML, the same European researchers who carried out the studies in B-CLL discussed earlier in this section (Minke et al., 2009.) treated the AML cell lines Kasumi-1 and HL-60, primary AML blasts, and healthy peripheral blood mononuclear cells with various concentrations of CGP049090 and PFK115-584.

Treatment with both compounds resulted in a significant killing of AML cell lines and primary AML blasts, with 50% effective concentration doses in the submicromolar range. PBMCs were not significantly affected. Killing of AML cells was mediated by apoptosis. Moreover, both compounds decreased expression of Wnt pathway downstream target genes such as c-Myc and cyclin D1.

In summary, Novartis researchers and their academic collaborators have concluded that the compounds CGP049090 and PFK115-584 warrant further studies to determine the feasibility of testing them in human clinical trials.

Novartis researchers have also been looking for other means to target the Wnt pathway, namely by performing chemical genetic studies to identify novel targets that modulate the Wnt pathway. They found that inhibition of the enzymes tankyrase 1 and tankyrase 2 with a “tool compound” resulted in stabilization of the destruction complex and resulted in increased degradation of β-catenin and inhibition of Wnt-pathway mediated transcription (Huang et al., 2009). Novartis researchers have been working to optimize their initial tool compound tankyrase inhibitors to produce lead compounds that could be entered into clinical trials. Thus Novartis has been working to find at least two alternative ways to target the Wnt pathway, a pathway that has resisted drug development up to now.

Small-molecule inhibitors of the BCL6/SMRT PPI in B-cell lymphoma

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin’s lymphoma, and accounts for about 30% of all lymphomas. In the United States, DLBCL affects about seven out of 100,000 people each year (Friedberg, 2011).

B-cell lymphoma 6 (BCL6) acts as an oncogene in the majority (40–70%) of cases of DLBCLs (Compton and Hebert, 2010). BCL6 protein is an evolutionarily conserved zinc-finger transcription factor. It has an N-terminal BTB (for BR-C, ttk, and bab) domain. (BTB domains are also known as POZ (for Pox virus and Zinc finger) domains.) BTB domains constitute a common class of homodimeric domains that are involved in PPIs. The BTB domains of BCL6 and other zinc finger proteins have been shown to mediate transcriptional repression. Via its BTB domain, BCL6 interacts with a corepressor known as SMRT (silencing mediator for

retinoid or thyroid-hormone receptors); SMRT is also known as nuclear receptor co-repressor 2 (NCOR2). SMRT in turn facilitates the recruitment of histone deacetylase 3 (HDAC3) to the DNA promoters bound by BCL6. This results in the repression of the genes controlled by the promoters, via removal of acetyl groups from histones of chromatin.

In normal lymphoid germinal center B-cell development, immunoglobulin genes undergo recombinations and somatic mutations to generate antibody diversity. These germinal center B cells are able to undergo rapid proliferation despite this genomic instability, because BCL6 represses a set of genes that regulate the DNA damage response and cell cycle checkpoints (Compton and Hebert, 2010). Among these genes are ATR (ataxia telangiectasia and Rad3-related protein), CHK1 (checkpoint kinase 1), TP53 (which codes for p53), and CDKN1A (cyclin-dependent kinase inhibitor 1). Once B-cell clonal diversity has been achieved, BCL6 expression is downregulated. This allows restoration of cell-cycle checkpoints and normal DNA damage control, and further B-cell differentiation and maturation. Oncogenic overexpression of BCL6 (which can occur via chromosomal translocation, gene amplification, or promoter mutation) results in continued B-cell progenitor proliferation and acquisition of additional mutations. This results in an aggressive B-cell lymphoma.

A group of academic researchers led by Ari Melnick (Weill Cornell Medical College, Cornell University, New York, NY) discovered small-molecule antagonists of the PPI between BCL6 and SMRT, and demonstrated that these compounds could kill DLBCL cells *in vitro* and in xenograft mouse models. This work was published in 2010 (Cerchietti et al., 2010).

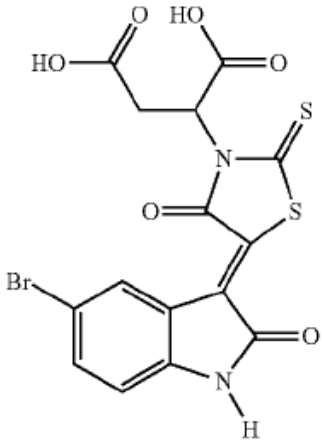
The researchers had been conducting X-ray diffraction structural studies of the interaction between BCL6 and SMRT since 2003 (Ahmad et al., 2003; Ghetu et al., 2008). They defined a minimal 17-residue fragment of SMRT (from leucine-1414 to arginine-1430). The researchers cocrystallized the BCL6 BTB domain with the 17-amino acid SMRT peptide and determined the structure. The peptide (and the SMRT protein) binds with micromolar affinity to the lateral groove of the BTB domain. The amino acid residues that form this lateral groove are not conserved in other transcription factors of the BTB family, which enables the possibility of discovering specific antagonists of the BCL6/SMRT PPI.

The researchers also identified via structural studies a small pocket within the BCL6 BBD lateral groove that associated with SMRT residues 1423–1428 and had a high complexity and density of contacts between the BTB and the SMRT peptide. Alanine scanning mutagenesis showed that all six BBD residues are required for the stability of the complex. This pocket thus constitutes a PPI hotspot, which might be suitable for binding of small-molecule antagonists of the BCL6/SMRT PPI.

The researchers therefore selected this region for application of computer-aided drug design (CADD), to first identify putative small-molecule binding sites, and then to use their model to screen 1,000,000 commercially available compounds by virtual docking of the compounds into the putative binding site (Cerchietti et al., 2010). Compound selection was based on both maximizing chemical diversity and optimizing properties associated with drug-like characteristics according to Lipinski's Rule of Five. The researchers thus built into their compound selection from the beginning provision for focusing on compounds that were most likely to be developable as human medicines.

The computer modeling study resulted in the identification of a set of 1,000 small molecules that were predicted to bind the BCL6 pocket. These compounds were organized by structural similarity into 100 groups, and one or two compounds were selected from each group based on the best drug-like properties. Of the nearly 200 compounds selected, 100 were immediately available from commercial vendors. These 100 compounds were then screened for their ability to block BCL6-mediated transcriptional repression using a cellular reporter assay. As a result of successive rounds of screening, a lead compound designated "79-6" (PubChem CID5721353), which reproducibly inhibited BCL6, was selected. The structure of this compound is shown in Figure 9. The researchers showed that 79-6 bound the target pocket of the BCL6 BTB domain, displaced the SMRT peptide from a complex with BCL6-BTB with a micromolar dissociation constant, and prevented the recruitment of SMRT/HDAC3 corepressor complexes to the ATR locus in cellular assays without affecting BCL6 binding to the DNA.

Figure 9: Chemical structure of 79-6



Source: US2012/0014979

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In accordance with the lack of conservation of amino acid residues that form the BTB lateral groove of BCL6, 79-6 did not affect repression of transcription caused by any other BTB-family zinc-finger proteins that were tested. The compound therefore appears to be selective for BCL6. Moreover, 79-6 only reactivated transcriptionally repressed genes in human DLBCL lines that expressed BCL6, not those that did not. 79-6 also specifically killed BCL6-expressing DLBCL lines that expressed BCL6 via apoptosis, but it had no effect on BCL6-independent DLBCL lines. The compound also induced apoptosis in primary human DLBCL cells that expressed BCL6.

Moreover, 79-6 suppressed the growth of tumors derived from BCL6-postive DLBCL cell lines in xenograft mouse models, and it induced apoptosis in the cells of these tumors. The compound was ineffective in reducing the tumor burden in xenograft mouse models bearing tumors derived from BCL6-negative DLBCL cell lines.

Compound 79-6 was nontoxic in mice. It also displayed favorable pharmacokinetics, as demonstrated by its ability to penetrate tumors after parenteral administration at a distal site. This result, coupled with the ability

of the compound to reduce tumor burdens and to induce apoptosis in BCL6-positive DLCBL tumors in mice, supports the idea of using 79-6 as a lead compound for development of a clinical candidate.

Currently, the researchers are working on optimizing 79-6 to develop a clinical candidate for BCL6-targeted therapy for DLBCL (Cerchietti et al., 2010). One issue is that the five-membered ring of 79-6 (Figure 9), which contains sulfur, may be prone to oxidation, which could result in loss of efficacy and more rapid clearance of the compound from the circulation. The researchers are investigating the extent of this oxidation, and are also attempting to replace the sulfur-containing ring with other heterocycles less prone to oxidation. Such modifications may also enhance oral availability.

BCL6/SMRT antagonists and the issue of targeting epigenetic regulation

As discussed in Chapter 1, analysts have identified epigenetic regulation as a potentially important area of opportunity for drug discovery. Because of its recruitment of a corepression complex that includes HDAC3, an enzyme involved in epigenetic silencing of chromatin, development of a PPI antagonist that disrupts this complex may provide a specific means of targeting this mode of epigenetic regulation. So far, two inhibitors of class I HDACs (class I HDACs include HDAC1, 2, 3, and 8) have been approved for the treatment of cutaneous T-cell lymphoma (CTCL) – Merck's vorinostat (Zolinza) and Gloucester/Celgene's romidepsin (Istodax) – and other agents are in clinical trials for various types of cancer. However, the mechanisms by which these compounds work, and why they appear to be active against certain cancers but not others and not normal cells, are unknown.

HDAC inhibitors have been studied in clinical trials against DLBCL as single agents (Compton and Hiebert, 2010). However, the results of Phase II trials of these agents have been disappointing. This may be related to the unknown nature of the mechanisms of action, and the specificities, of these compounds. It is possible that a more selective HDAC3 antagonist might give better results, as suggested by Compton and Hiebert (Compton and Hiebert, 2010). However, such an agent would still be subject to the many unknowns surrounding the mechanisms of action and specificities of HDAC inhibitors. Exquisitely specific agents that modulate the proteins that recruit HDACs to their sites of action, as represented by 79-6 targeting of BCL6, may be a better solution.

Moreover, in the case of DLBCL, a BCL6/SMRT PPI antagonist could be used to treat the disease in combination with rituximab (Biogen Idec/Genentech's Rituxan). Rituximab, which targets the B-cell-specific cell-surface protein CD20, is currently used in combination with chemotherapy to treat DLBCL. Although this combination therapy results in a cure in over half of all patients, there is still a large fraction of patients who have refractory or recurrent disease. These have a more unfavorable prognosis. A drug that specifically targets the BCL6/SMRT PPI, perhaps used in combination with rituximab, may address the unmet medical needs represented by patients with refractory or recurrent disease and may reduce the need for toxic chemotherapy in at least a large fraction of DLBCL patients.

As we mentioned in Chapter 2, Forma Therapeutics, in collaboration with the Leukemia & Lymphoma Society, is also working on the discovery and development of inhibitors of the BCL6/SMRT PPI. Thus there is wider interest in developing such agents for the treatment of DLBCL.

Small-molecule AKAP-protein kinase A interaction disruptors for potential treatment of chronic heart failure

A-kinase anchoring proteins (AKAPs) are scaffolding proteins that tether protein kinase A (PKA) and other signaling proteins to specific intracellular sites. PKAs (also known as cAMP-dependent protein kinases) are a family of serine/threonine kinases whose activity is dependent on cellular levels of cyclic AMP (cAMP). Thus the tethering of PKA via a PPI with an AKAP results in the compartmentalization of cAMP signaling within the cell.

Scaffolding proteins represent important means by which the cell organizes signal transduction pathways. For example, the scaffolding protein known as kinase suppressor of RAS (KSR) acts as a scaffold to assemble the well-known RAS-RAF-MEK-ERK pathway, whose dysregulation results in a wide array of human cancers (Clapéron and Therrien, 2007). In most cases, scaffolding proteins act via forming PPIs with the signaling proteins that they organize. Thus, discovering small-molecule inhibitors of these PPIs is a potential strategy for targeting a wide array of signaling pathways.

AKAP18δ is an AKAP isoform that serves as a scaffold for organizing the adrenaline-beta-adrenoreceptor-cAMP-PKA signaling pathway in cardiac muscle (Lygren and Taskén, 2008). This pathway regulates heart

rate and contractility by adrenergic control of calcium reabsorption into the sarcoplasmic reticulum of cardiac muscle cells. Calcium reabsorption mediates relaxation and filling of the heart and is the rate-limiting step for increasing heart rate in response to adrenaline or noradrenaline. Changes in contractility are associated with cardiovascular disease, especially heart failure. Existing drugs, such as beta-blockers, are ineffective in modulating this system. Researchers would therefore like to discover and develop drugs that modulate the cardiac myocyte cAMP-PKA system. Targeting AKAP18δ and its PPI with PKA is a potential approach to accomplishing this.

Researchers at the Leibniz Institute for Molecular Pharmacology (Berlin, Germany) (FMP) and their colleagues have been conducting studies aimed at discovering small-molecule disrupters of AKAP18δ-PKA interactions (Christian et al., 2010). This research was facilitated by the FMP Medicinal Chemistry group's program for the discovery of PPI inhibitors and by its design and construction of chemical libraries (Leibniz-Institut für Molekulare Pharmakologie, 2011). As part of these programs, the FMP Medicinal Chemistry group has also been involved in the development of biochemical assays that are used in the FMP PPI inhibitor program.

The PKA holoenzyme consists of a dimer of regulatory (R) subunits (RIα, RIβ, RIIα, or RIIβ) and two catalytic subunits (Ca, Cb, or Cg), each of which is bound to an R subunit (Christian et al., 2010). RI-containing holoenzyme is called PKA type I, whereas RII-containing PKA is termed PKA type II. Binding of cAMP to the R subunits results a conformational change, which causes release and activation of the catalytic subunits, which then phosphorylate various substrates.

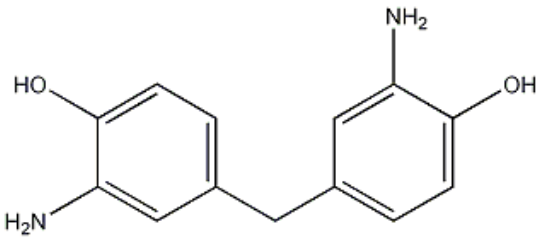
AKAPs, which control the intracellular localization of PKA, possess an RII-binding domain by which they bind to PKA holoenzyme. This binding domain is an amphipathic α-helical structure that consists of 14–18 amino acids. The domain interacts with the hydrophobic groove formed by the N-terminal dimerization and docking (D/D) domain of regulatory subunit dimers. Besides PKA, AKAPs can bind other signaling proteins. This enables these scaffolding proteins to coordinate entire signaling pathways (for example, the adrenaline-beta-adrenoreceptor-cAMP-PKA signaling pathway) and to localize them within the cell.

To screen for AKAP-PKA interaction disruptors, the FMP researchers established an ELISA-based screening assay in which full-length AKAP18δ was added to RIIα subunits of PKA bound to 384-well microtiter plates.

Interaction was detected using AKAP18δ-specific Abs, secondary peroxidase-conjugated antibodies, and a chemiluminescent peroxidase substrate. This assay was used to screen an FMP chemical library known as FMP_20.000, which contained 20,064 compounds. The molecules in this library had an average molecular mass of 250 Daltons and were deemed to be drug-like based on the Lipinski rules. The library was designed to have a large chemical diversity.

As a result of this screen, nine compounds were identified as potential disruptors of the AKAP18δ-RIIa PPI. The most promising of these compounds, 3',3-diamino-4,4'-dihydroxydiphenylmethane (CAS 16523-28-7) was designated as FMP-API-1. The structure of this compound is shown in Figure 10.

Figure 10: Chemical structure of FMP-API-1



Source: www.chemicalbook.com

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FMP-API-1 was validated as an AKAP18δ-RIIa PPI antagonist via a secondary assay, which was a competition assay between the compound and the peptide AKAP18δ-L314E (i.e. a peptide derived from the RIIa-binding domain of AKAP18δ). The peptide was immobilized on the surface of a surface plasmon resonance (SPR) sensor chip, and binding of RIIa subunits with or without preincubation with FMP-API-1 or other test compounds was measured using a Biacore 3000 SPR instrument. FMP-API-1 inhibited the interaction with a micromolar dissociation constant. The researchers also investigated derivatives of FMP-API-1, performing preliminary structure-activity relationship (SAR) studies.

The researchers used Biacore sensor chip-based SPR assays to determine whether FMP-API-1 and its derivatives acted via an allosteric mechanism, or directly at the D/D domain by which RIIa binds to AKAP18δ or peptides derived from its RIIa-binding domain. Sensor chips were coated with full-length RIIa, and the association and dissociation of FMP-API-1, two of its derivatives, and each small molecule combined with the peptide AKAP18δ-L314E was measured. The association curve resulting from the combination of small

molecule and peptide suggested binding of the small molecules to an allosteric site on RIIa rather than to the D/D domain. The researchers therefore coupled the D/D domain to the sensor chip, to investigate whether it would bind FMP-API-1 or its derivatives. It did not, confirming that FMP-API-1 must act at an allosteric site on the RIIa regulatory subunit. NMR studies confirmed that FMP-API-1 bound to RIIa in a noncovalent and reversible manner. Further SPR studies with truncated versions of RIIa also indicated that the binding site on RIIa was located C-terminal to the D/D domain. Further studies of the nature of the allosteric site would be greatly facilitated by obtaining a three-dimensional structure for RIIa via X-ray diffraction. However, unfortunately, attempts to obtain such structures for full-length RII subunits have been unsuccessful.

Biochemical studies showed that FMP-API-1 did not activate catalytic subunits of PKA (i.e. PKA in the absence of regulatory subunits), nor did it activate other kinases that are involved in controlling cardiac myocyte function. However, FMP-API-1 did activate PKA in intact cardiac myocytes, to a similar extent to the beta-adrenergic agonist isoproterenol. Activation of PKA by FMP-API-1 and isoproterenol was additive.

The researchers found via electrophysiological measurements that FMP-API-1 inhibited β-adrenoreceptor-mediated increases of L-type calcium and IKs potassium channel currents in rat neonatal cardiac myocytes. In both cases, AKAP18δ recruits PKA to the respective channels, and PKA-mediated phosphorylation of the respective channels results in enhanced entry of calcium and potassium ions, respectively, into cardiac myocytes (Christian et al., 2010). Thus FMP-AP-1 appears to inhibit the action of these channels via the disruption of AKAP18δ/PKA PPI.

The researchers also showed that FMP-AP-1 increased the contractility of cultured myocytes and isolated rat hearts (Christian et al., 2010). More specifically, FMP-AP-1 increased rates of cardiac myocyte shortening and of re-lengthening by 15–20% compared with a solvent control (0.1% DMSO). In isolated perfused hearts, FMP-API-1 caused a small but significant increase of 16% in the rate of left-ventricular pressure development, of 13% in the contraction rate and of 18% in the maximal relaxation rate. FMP-API-1 administration thus appears to produce both positive inotropic (i.e. increases the rate of cardiac contraction) and lusitropic (increases the rate of cardiac contraction) effects on the perfused heart by its effects on organization of cAMP/PKA signaling by AKAP.

Currently approved positive inotropic drugs (e.g. beta-adrenergic agonists such as isoproterenol, phosphodiesterase inhibitors such as enoximone (Sanofi's Perfan)) may be initially effective in treating heart failure, but long-term treatment with these agents harm the failing heart. This may occur because these agents constitutively and nonspecifically activate PKA throughout the cardiac myocyte. In contrast, specific disruption of AKAP/PKA interactions has a local effect on specific pathways within the cell. The researchers who carried out this study (Christian et al., 2010) speculate that this local action may lead to more effective positive inotropic (and lusitropic) treatments with fewer adverse effects. More generally, they (and others) speculate that specific PPI modulators may produce highly specific pharmacological interference with defined cellular processes and thus constitute better drugs than agents that do not target PPIs.

However, these researchers realize that FMP-API-1 represents only a starting point for the development of higher affinity drug-like small-molecule AKAP-PKA PPI antagonists. Such compounds, once they are developed, might be advanced into the clinic for the treatment of chronic heart failure, a disease with a high unmet medical need. In the meantime, FMP-API-1 might be used as a tool compound to investigate the function of AKAP-PKA PPIs in cells and in animal models. Moreover, as discussed earlier, scaffolding proteins are important in organizing many signal transduction pathways and may provide numerous opportunities for targeting by small-molecule PPI modulators.

Conclusions

As shown by these three case studies, researchers have found that it is possible to target PPIs involved in intracellular signaling pathways with small-molecule agents, and at least in some cases to discover compounds that can be taken into the clinic. In the first case study, a pharmaceutical company (Novartis) is already following up on research from an academic group and has been conducting late preclinical studies, which may result in an investigational new drug (IND) application. In the second case study, academic researchers are performing lead optimization that may result in a developable compound, provided they can either find an industry partner or spin out a biotech company. Meanwhile, Forma Therapeutics intends to develop compounds that address the same PPI. In the third case study, academic researchers have discovered drug-like compounds that address what appears to be a therapeutically important PPI target, but they need to optimize the chemical matter prior to entering preclinical studies with the intent of developing a

clinical compound. Meanwhile, they can use the compounds they have discovered as tool compounds to probe the mechanism further.

There are at least several other academic groups that are investigating small-molecule modulators of PPIs involved in signaling pathways, and these groups have produced tool compounds that for various reasons (pharmacokinetics, stability, etc.) cannot become drugs. However, such compounds can be useful in exploring signaling pathways and qualifying the targets of these compounds as potential targets for safe and effective drugs. And with the types of new technologies discussed in Chapter 2, more of these academic groups may be able to translate their research efforts into the discovery of developable drugs. Notably, both research groups in the last two case studies discussed in this chapter have been paying special attention to designing libraries of compounds with “drug-like” properties, so they have been thinking about the issue of eventual developability. The addition of libraries of macrocycles (as discussed in Chapter 2) may increase the likelihood of discovering developable compounds, for these and other researchers. And as shown in the third case study, the discovery of allosteric modulators of PPIs may also provide the means of discovering drug-like compounds in cases in which it is difficult to discover compounds that directly modulate a PPI.

Research aimed at the discovery of small-molecule modulators of PPIs involved in intracellular signaling is in most cases in the hands of academic researchers. However, in the current era with large pharmaceutical companies increasing their emphasis on acquiring external R&D (including from academia) while cutting internal R&D, this area of research may provide opportunities for both industry and academia.

Chapter 5 Small molecules targeting the ubiquitin system

Summary

- The ubiquitin system is a fundamental regulatory system in all eukaryotic cells, comparable in importance to protein phosphorylation. This system is best known for regulating intracellular protein turnover but is also involved in such functions as regulation of mitosis and innate immunity, and regulation of certain protein kinases and other enzymes.
- The ubiquitin system is based on covalently linking the small (8.5kDa) regulatory protein ubiquitin – either singly or as polyubiquitin chains – to numerous specific protein targets. In protein degradation the ubiquitin system works together with the proteasome in a pathway known as the ubiquitin proteasome system (UPS). In the UPS, ubiquitin is used to tag proteins for degradation by the proteasome.
- Many researchers see the ubiquitin system as a virtually untapped area of opportunity for drug discovery and development, potentially comparable in importance to protein and lipid kinases. The first approved drug, the proteasome inhibitor bortezomib (Millennium's Velcade), was approved in 2003.
- The ubiquitinylation pathway is complex and involves several levels of mediators, principally E1 ubiquitin activators, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. In this pathway, ubiquitin moves from E1s to E2s, and E3s interact via PPIs with ubiquitinylated E2s and with substrate proteins. In these complexes, ubiquitin is transferred from the E2 to the substrate. This process may be repeated, resulting in tagging of substrates with polyubiquitin chains.
- As one moves down the ubiquitinylation cascade, the degree of specificity of the process, and therefore the potential for development of specific drugs, increases. There are only 10 E1s encoded in the human genome, but there are about 40 E2s and over 600 E3s.
- Drugs in clinical development that target the UPS include second-generation proteasome inhibitors, and two agents that target the ubiquitinylation pathway itself. These include Millennium's MLN4924, which inhibits a pathway that activates one class of E3s, the Cullin RING E3 ligases (CRLs), and

Celgene's CC0651, an allosteric modulator that inhibits the ubiquitinylation activity of an E2 that interacts with CRLs. These agents, which are both enzyme inhibitors, appear to be more specific than proteasome inhibitors, but their targets ubiquitinylate hundreds of substrate proteins.

- The most specific agents that target the ubiquitin system would be agents that target E3s. Since E3s interact with their substrates via PPIs, such agents would be PPI modulators. Thus the intractability of PPIs has been a major bottleneck to the development of specific agents that target the ubiquitin system.
- Researchers have developed agents that target one E3. This is HDM2, which is the human homolog of mouse double minute 2 (MDM2) protein. HDM2 interacts with p53 via a PPI. p53, the "guardian of the genome," controls pathways that respond to DNA damage or other insults by blocking cell proliferation, and then either inducing DNA repair or inducing apoptosis.
- p53 is mutated or inactivated in nearly all human cancers, which allows these cancers to proliferate continuously and renders them resistant to cytotoxic chemotherapy. In approximately 50% of human cancers, p53 is inactivated via mutation. In the other 50%, p53 remains unmutated, but is inactivated. The main means of inactivation is via HDM2, which is overexpressed in the majority of cancers with wild type p53.
- HDM2 regulates p53 in three ways: inhibition of p53-induced transcription, promotion of export of p53 out of the nucleus, and inducing p53 degradation by the proteasome. Both of the latter activities involve HDM2's E3 ubiquitin ligase activity.
- There are currently two leading drug candidates that specifically disrupt the HDM2/p53 PPI. The most advanced compound, now in Phase I clinical trials, is Roche's RG7112, which is an analog of nutlin-3a. The other compound is Ascenta/Sanofi's MI-219, analogs of which are currently in advanced preclinical studies.
- Johnson & Johnson's JNJ-26854165, which is in Phase I clinical trials in advanced or refractory solid tumors, was an agent with an unknown mechanism of action that had been deemed to target the HDM2/p53 PPI. However, recent studies indicate that it works via a different mechanism of action that does not involve HDM2.

- Given the large number and specificity of E3 ubiquitin ligases and their important role in intracellular pathways, there is a large field of possibility for discovery of novel PPI modulators that target these biomolecules and their interactions with their substrates.

Introduction

The ubiquitin system is a fundamental regulatory system in all eukaryotic cells, comparable in importance to protein phosphorylation (Cohen and Tcherpakov, 2010). This system is best known for regulating intracellular protein turnover. However, the ubiquitin system is also involved in such functions as regulation of mitosis and innate immunity, and regulation of certain protein kinases and other enzymes (Cohen and Tcherpakov, 2010).

The ubiquitin system is based on covalently linking the small (8.5kDa) regulatory protein ubiquitin – either singly or as polyubiquitin chains – to numerous specific protein targets. The regulatory function of ubiquitin in protein turnover, and the components of the ubiquitination pathway, were determined in the early 1980s by Aaron Ciechanover, Avram Hershko, and Irwin Rose, who were awarded the Nobel Prize in Chemistry in 2004 for this work (Nobel Prize in Chemistry, 2004). In protein degradation, the ubiquitin system works together with the proteasome. Proteasomes are very large protein complexes that degrade unneeded or damaged proteins via the action of proteasomal proteases. The ubiquitinylation pathway working together with the proteasome is known as the ubiquitin proteasome system (UPS).

In the UPS, ubiquitin is used to tag proteins for degradation by the proteasome. Via a series of enzymatic steps, first a single ubiquitin is attached to the protein, and then additional ubiquitins are attached to the original one, resulting in a polyubiquitin chain. Polyubiquitinylated proteins are recognized by the proteasome, which then degrades these tagged proteins.

The UPS is part of the network of pathways within the cell that maintains protein homeostasis, or “proteostasis.” Proteostasis pathways control the biosynthesis, folding, trafficking, and turnover of intracellular and extracellular proteins.

Several analysts and companies see the ubiquitin system as a virtually untapped area of opportunity for drug discovery and development. In particular, a November 2010 Leading Edge Perspective article in Cell

compares the field of protein ubiquitinylation in importance to that of protein phosphorylation (Cohen and Tcherpakov, 2010). The protein phosphorylation field (exemplified by protein kinases and phosphatases; there are lipid kinases and phosphatases not mentioned in the article that are also part of this regulatory system) resulted in a 1992 Nobel Prize; however, kinases were thought to be poor targets for a long time. Nevertheless, in the late 1990s Novartis and its academic collaborators developed the first kinase inhibitor, imatinib (Gleevec/Glivec), which was approved in 2001. This was followed by a flood of kinase inhibitors, both approved and still moving through development.

In the case of the ubiquitin pathway (exemplified by ubiquitin ligases and deubiquitinases, i.e. enzymes that attach and detach ubiquitin to proteins), the first approved drug, bortezomib (Millennium's Velcade), was approved in 2003. However, bortezomib, a proteasome inhibitor, remains the only approved drug, although 16 others were reported to be in the clinic as of late 2010 (Cohen and Tcherpakov, 2010). As will be discussed later in this chapter, the lag in development of ubiquitin system modulators is due in large part to the intractability of PPIs.

At least two companies are focusing a major part of their R&D efforts on proteostasis, and the ubiquitin system in particular. Millennium Pharmaceuticals, The Takeda Oncology Company (Cambridge, MA), is the developer of bortezomib. It sees proteostasis as an important area of R&D, and is actively developing compounds that disrupt the UPS, all of which are enzyme inhibitors. Meanwhile, a research-stage biotech company founded in 2008 known as Proteostasis Therapeutics (Cambridge, MA), is developing therapeutics that address several pathways in the proteostasis network – not only protein clearance via the UPS, but also protein folding and trafficking. With respect to the UPS, Proteostasis licensed several novel targets and small-molecule compounds from Harvard University in 2011 (Proteostasis Therapeutics, 2011).

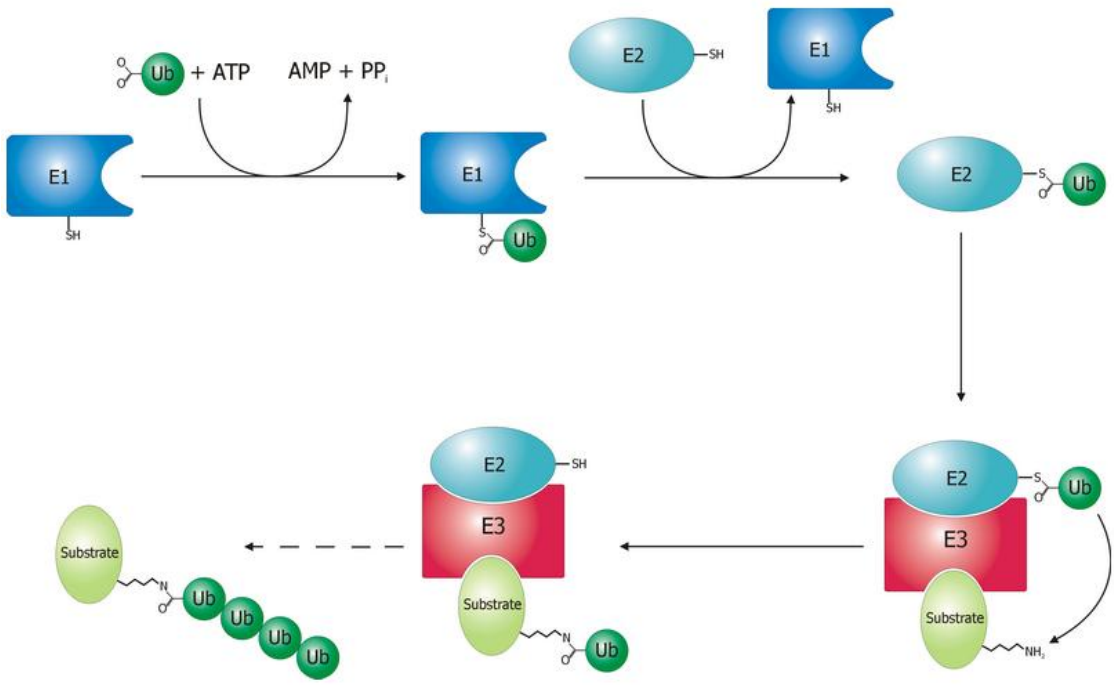
As will be discussed later in this chapter, several other companies are pursuing more targeted programs, each of which addresses one aspect of the UPS, in some cases involving targeting of PPIs.

The ubiquitin system

Figure 11 is a schematic diagram of the ubiquitinylation pathway. As show in the figure, the first step of the ubiquitinylation cascade is the ATP-driven attachment of ubiquitin to a ubiquitin-activating enzyme, known as

an E1. In this process, the ubiquitin is attached via a thioester bond between the C-terminal carboxylate group of ubiquitin and the sulfhydryl group of a cysteine residue on the E1 (Cohen and Tcherpakov, 2010; Deshaies, 2009). In the second step of the cascade, the activated ubiquitin is transferred from the E1 to a cysteine on a ubiquitin-conjugating enzyme, known as an E2. In the third step, the E2 with its activated ubiquitin interacts with a ubiquitin ligase, known as an E3. The E3 also interacts with a specific substrate protein. In this complex, the ubiquitin is transferred from the E2 to the substrate, via several mechanisms. With some E3s, such as the Cullin RING E3 ligases (CRLs), the ubiquitin is transferred directly from the E2 to the substrate as shown in Figure 11. (This is the largest family of E3 ligases in the human genome, with over 100 members; as discussed later in this section, they must be activated by neddylation to carry out their function.) In other cases, such as the HECT family of E3 ligases, the ubiquitin is transferred first to a cysteine on the E3, and then to the substrate. Ubiquitin is linked to the substrate covalently, via a peptide bond. Polyubiquitin chains are formed via the same process.

Figure 11: The ubiquitinylation pathway



Source: Liljas, 2009

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In addition to ubiquitin, there are several classes of ubiquitin-like proteins, including Nedd8, SUMO, FAT10, and ISG15. These are attached covalently to substrate proteins via processes that are similar to ubiquitinylation. The processes are called, respectively, neddylation, SUMOylation, tenylation, and ISGylation (Cohen and Tcherpakov, 2010).

As with phosphorylation, ubiquitinylation is reversible, via a class of isopeptidase enzymes called deubiquitinases or DUBs. These enzymes catalyze the cleavage of the ubiquitin from proteins.

As one moves down the ubiquitinylation cascade, the degree of specificity of the process, and therefore the potential for development of specific drugs, increases. There are only 10 E1s encoded in the human genome, but there are about 40 E2s and over 600 E3s (Cohen and Tcherpakov, 2010). There are also approximately 90 deubiquitinases, which is comparable to the number of protein phosphatases. However, the total number of E1-activating enzymes, E2-conjugating enzymes, and E3 ligases encoded by the human genome is greater than the number of protein kinases.

Proteasome inhibitors and the ubiquitin-proteasome system

The issue of drug discovery and the specificity of targets at different levels of the ubiquitinylation cascade are illustrated by considering the approved drug bortezomib, and several pipeline drugs that target enzymes in the cascade. Bortezomib does not target any member of the ubiquitinylation cascade, but targets protease activity of the proteasome, the organelle that disposes of polyubiquitinylated proteins. Based on this mechanism of action, bortezomib is highly nonspecific, blocking proteasomal disposal of all ubiquitinylated proteins in the cell.

However, despite this nonspecificity, bortezomib is still a useful drug. In multiple myeloma (MM), malignant plasma cells undergo massive proliferation, and in the process create large amounts of damaged proteins. These must be cleared by the proteasome. However, in MM cells, proteasomes can barely keep up with the overload of damaged proteins. Blocking the proteasome with bortezomib results in an overload of damaged proteins, which destroys the cell (apparently via apoptosis) (Appel, 2011).

However, as might be expected for a drug with such a nonspecific mechanism, bortezomib has severe adverse effects. The most common adverse effect is nausea. The drug is also associated with peripheral

neuropathy, as well as myelosuppression leading to neutropenia and thrombocytopenia (i.e. deficiencies in neutrophils and thrombocytes). The drug is also associated with toxicities in the heart, lungs, and kidneys. Although these adverse effects are generally less severe than those associated with such alternative treatment options as bone marrow transplantation, many patients discontinue bortezomib treatment before completing a full course.

Some researchers attribute bortezomib's adverse effects to its nonspecific inhibition of proteasomal enzyme activities (Appel, 2011). Although the specific target of bortezomib is the β5 chymotrypsin-like enzyme activity of the proteasome, it can also disrupt the two other proteasomal enzymes, β1 and β2. Moreover, as with most oncology drugs, nearly all patients develop bortezomib resistance.

As a result, several companies are developing second-generation proteasome inhibitors (Appel, 2011). Among these is Millennium's MLN9708, which, unlike bortezomib and other proteasome inhibitors, is an oral drug. MLN9708 is more specific for the chymotrypsin-like proteasomal enzyme than is bortezomib, and it shows a low association with neuropathy. MLN9708 is in Phase I and Phase II trials in MM patients who have relapsed on bortezomib, and in combination with other drugs for newly diagnosed patients. It appears to be effective in overcoming bortezomib resistance.

The most advanced second-generation proteasome inhibitor in development is Onyx Pharmaceuticals' (South San Francisco, CA) carfilzomib, which is in Phase III trials. Carfilzomib is a more selective inhibitor of the proteasomal chymotrypsin-like protease enzyme than is bortezomib, and less than 1% of patients experience neuropathy on this drug.

Other companies developing second-generation proteasome inhibitors include Nereus Pharmaceuticals (San Diego, CA) and Cephalon (Frazer, PA).

Ubiquitin-proteasome system inhibitors of intermediate specificity

Millennium has another drug in clinical trials that targets an arm of the ubiquitinylation cascade itself. (Deshaies, 2009; Soucy et al., 2009). The company is developing MLN4924, an AMP analog that inhibits NEDD8-activating enzyme (NAE).

NEDD8 is a ubiquitin-like protein, and NAE is an E1 for neddylation. As with the canonical E1s for ubiquitinylation, NAE requires ATP for its activity, and releases AMP once it attaches NEDD8 to itself via a thioester bond (hence the inhibition of NAE by the AMP analog MLN4924). NAE then transfers NEDD8 to an E2 for neddylation, known as a NEDD8 conjugating enzyme (N8 E2). This enzyme then transfers NEDD8 to a specific site on a member of the class of E3s for ubiquitinylation know as a CRL. When NEDD8 is bound to a CRL that interacts with an ubiquitinylated E2 and a substrate protein, the CRL changes its conformation in such a way as to bring the E2 in proximity to the substrate, enabling the E2 to ubiquitinylate the substrate.

Inhibition of neddylation by MLN4924 at the E1 (NEDD8-activating) step thus indirectly inhibits one class of E3s, the CRLs, without the need to inhibit the PPIs between members of this class of E3s and the substrate proteins that bind to them, or the need to inhibit the catalytic activity of E2s or the PPIs between E2s and the CRL E3s. MLN4924 is thus much more specific than bortezomib in inhibiting the ubiquitin system. However, since it inhibits an entire class of E3s with over 100 members in humans, it is nowhere as specific as, for example, a drug that inhibits a PPI between a specific substrate and a specific E3. The Millennium researchers, on the basis of studies with MLN4924, estimated that in HCT-116 colorectal carcinoma cells, approximately 20% of proteasome-dependent protein degradation is mediated by CRL ubiquitinylation. (Deshaies, 2009; Soucy et al., 2009). This shows that, despite the greater degree of specificity of MLN4924 compared with a proteasome inhibitor like bortezomib, MLN4924 is not very specific with respect to targeting proteins for degradation.

Nevertheless, *in vitro* and preclinical studies indicate that MLN4924 has potentially important antitumor effects (Deshaies, 2009; Soucy et al., 2009). MLN4924 treatment of human tumor cells *in vitro* induced apoptosis via uncontrolled DNA synthesis in the S-phase of the cell cycle, leading to DNA damage and induction of apoptosis. MLN4924 also suppressed the growth of human tumor xenografts in mice, and appeared to be well tolerated. MLN4924 is now in Phase I clinical trials in patients with solid and hematological tumors.

Another approach to indirectly inhibiting CRLs, this time at the E2 level, was developed by researchers at the Celgene Signal Research Division (San Diego, CA) and their academic collaborators (Ceccarelli et al., 2011). These researchers discovered an allosteric inhibitor of the E2 ubiquitin-conjugating enzyme Cdc34.

Ubiquitinylated Cdc34 interacts with members of the CRL superfamily of C3 enzymes and then works with the CRLs to ubiquitinylate hundreds of substrate proteins, each of which also interacts with a CRL as described earlier. The allosteric Cdc34 inhibitor CC0651 inserts itself into a binding pocket of Cdc34 that is distinct from the catalytic site, causing a conformational change that results in inactivation of the catalytic activity of the E2. CC0651 analogs inhibited proliferation of human cancer cell lines. They also caused accumulation of the cyclin-dependent kinase (CDK) inhibitor p27Kip. p27Kip is a substrate protein of the Skp2 subunit of the multisubunit CRL E3 known as Skp1-Cdc53/ Cullin-F box protein (SCF).

Dysregulation of the ubiquitinylation of p27Kip would be expected to cause uncontrolled DNA synthesis in the S-phase of the cell cycle, leading to DNA damage and induction of apoptosis, similar to the action of MLN4924. The Celgene researchers and their collaborators are developing strategies for using CC0651 as the basis for cancer therapies, especially targeting cancers that exhibit overexpression of Cdc34. This especially includes T-cell acute lymphoblastic leukemia. Since a dominant negative allele of Cdc34 was found to enhance the efficacy of bortezomib against MM, that cancer may also be a target.

The E2 inhibitor CC0651 and its analogs appear to be more specific than MLN4924, which inhibits the activity of all CRLs. Nevertheless, the target of CC0651 still ubiquitinylates hundreds of substrate proteins, so it is relatively nonspecific.

Development of specific inhibitors of E3s

In the review by Cohen and Tcherpakov (2010) the authors discuss the question as to why drug discovery in the ubiquitin system has lagged way behind that of protein kinases. With respect to protein kinases, it has been easy to construct and screen large and relatively diverse kinase-focused chemical libraries, and to thus develop inhibitors of many protein kinases. Moreover, the targets in the receptor tyrosine kinase subclass have extracellular domains that have been targeted with mAb drugs.

Although there are more E3 ubiquitin ligases than kinases, researchers have not developed a general approach for identifying inhibitors of many of these biomolecules. Interactions between E3 ubiquitin ligases and their substrates are PPIs (see Figure 11) and have thus been deemed undruggable. Another issue, not mentioned in the review, is that it is necessary to first understand something of the biology and disease relevance of a substrate protein, and to identify the E3 with which it interacts. The authors of the review also

ask why more attention has not been given to developing compounds that disrupt the interactions between E2-conjugating enzymes and E3 ligases, since these PPIs are usually relatively weak and may therefore be easier to disrupt than PPIs between E3s and substrate proteins.

Subsequent sections of this chapter discuss efforts to develop small-molecule inhibitors of PPIs between specific E3s and specific substrate proteins, in cases in which particular progress has been made toward developing compounds with therapeutic potential.

Small-molecule antagonists of the HDM2/p53 PPI

The E3 ubiquitin ligase that has received the most attention from researchers, with the most successful results, is HDM2. HDM2 is the human homolog of MDM2, or mouse double minute 2 (MDM2) protein. Many papers refer to HDM2 as MDM2, which can sometimes be confusing. However, as discussed later, because of the high degree of conservation between mouse and human amino acid sequences involved in the interaction site for these two proteins, findings of studies of MDM2/p53 apply to HDM2/p53.

HDM2/MDM2 functions as an inhibitor of the tumor suppressor protein p53. Often called the “guardian of the genome,” p53 protects the organism from the effects of DNA damage, especially the induction of cancer. Acting as a transcriptional activator, it controls pathways that respond to DNA damage or other insults by blocking cell proliferation, and then either inducing DNA repair or inducing apoptosis. HDM2 carries out its p53-inhibitory function by binding to p53, thus forming a PPI.

Given the importance of p53 and HDM2/MDM2 as targets in oncology, much basic and drug discovery research has been expended on this system since the identification of the p53 protein in 1979. Two reviews (Shangary and Wang, 2009; Cheok et al., 2011) have recently been published, focusing on therapeutics that target this system and that are in the preclinical and early clinical stage of development. One review (Shangary and Wang, 2009) focuses exclusively on small-molecule compounds that target the HDM2/p53 PPI, while the other review (Cheok et al., 2011) has this field as its major focus.

p53 is mutated or inactivated in nearly all human cancers, which allows these cancers to proliferate continuously and renders them resistant to cytotoxic chemotherapy. In approximately 50% of human cancers, p53 is inactivated via mutation. In the other 50%, p53 remains unmutated, but is inactivated by

other means, mostly via HDM2. The gene for HDM2 can be amplified, leading to overexpression of HDM2 protein (Cheok et al., 2011). Alternatively, the promoter of the p14ARF gene may be methylated, resulting in the downregulation of p14ARF, a cell cycle regulatory protein that inhibits HDM2 expression. In the case of the approximately 5% of cancers (such as cervical cancer) that are induced by human papillomavirus, E6-associated protein (E6-AP) is expressed within these cells. E6-AP is itself an E3 ubiquitin ligase that targets p53. This results in the polyubiquitination of p53 and its disposal via the UPS.

In normal, unstressed cells, MDM2/HDM2 works to keep p53 under control, so that it does not interrupt normal cell physiology but is ready to act in case of DNA damage or other stresses. In an autoregulatory feedback loop, p53 activates HDM2 expression, which leads to HDM2-mediated p53 inhibition (Shangary and Wang, 2009). HDM2 regulates p53 in three ways: inhibition of p53-induced transcription, promotion of export of p53 out of the nucleus, and inducing p53 degradation by the proteasome. Both of the latter activities involve HDM2's E3 ubiquitin ligase activity. Monoubiquitinylation results in nuclear export (Li et al, 2003), while polyubiquitinylation results in proteasomal degradation (Shangary and Wang, 2009).

Given the complexity of coregulation of p53 and HDM2, it will be necessary to develop sets of biomarkers and diagnostic tests to guide therapy with HDM2/p53 PPI inhibitors and other drugs that are designed to modulate the HDM2/p53 system. Such diagnostic tests may include tests for TP53 mutational status and for HDM2 protein levels, mRNA levels, and gene copy number, as well as the status of other biomolecules (discussed below) that affect the HDM2/p53 system. Tests for TP53 mutational status are already the most common p53-related intervention in clinical trials (Cheok et al., 2011); such tests are usually used to stratify patients for therapy in trials of agents whose efficacy might be affected by TP53 mutational status. (As discussed earlier, mutated or inactivated p53 renders cancers resistant to cytotoxic drugs.)

Structural studies of the HDM2/p53 PPI

The nature of the interaction between HDM2 and p53 was established by the mid-1990s; since the structure and the amino acid residues involved are highly conserved between mice, humans, and even the frog *Xenopus laevis*, these results apply to MDM2/p53 as well (Kussie et al., 1996; Shangary and Wang 2009). Mutagenesis and X-ray diffraction structural studies showed that the MDM2/p53 interaction site maps to the N-terminal domain of HDM2 and the N-terminus of the transactivation domain of p53 (i.e. the domain that

induces transcription of downstream genes controlled by p53). The PPI involves a small, deep hydrophobic pocket in HDM2, and a short amphipathic (i.e. containing both polar and nonpolar amino acid residues) pocket in p53 that contains three critical hydrophobic residues (phenylalanine 19, tryptophan 23 and leucine 26). Unlike most PPIs, which involve a large binding interface, the HDM2/p53 binding interface is small and well defined. These properties of the interface gave encouragement to researchers who wished to discover or design small-molecule nonpeptide HDM2/p53 PPI inhibitors, and provided the basis for such efforts. However, it took several years before researchers were successful in discovering such inhibitors.

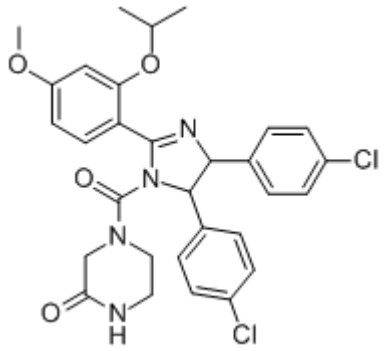
Nutlins

There are currently two leading drug candidates that specifically disrupt the HDM2/p53 PPI. The most advanced compound, now in Phase I clinical trials, is Roche's RG7112, which is an analog of nutlin-3a (Shangary and Wang, 2009).

The report of the research that resulted in the discovery of the nutlin series, named for the Roche research laboratories in which they were discovered in Nutley, NJ, was published in 2004 (Vassilev et al., 2004; Shangary and Wang, 2009). The Roche researchers discovered these compounds by screening a diverse chemical library using a Biacore surface plasmon resonance assay of the type described in Chapter 4, and by then optimizing lead compounds for potency and selectivity.

The nutlins discovered in these studies are series of cis-imidazoline analogs, named nutlins 1–3, respectively. The most potent compound, nutlin-3, had an IC_{50} (half-maximal inhibitory concentration) of 90 nanomolar. Nutlin-3a is the active enantiomer isolated from racemic nutlin-3. The structure of nutlin-3 is shown in Figure 12.

Figure 12: Chemical structure of nutlin-3



Source: www.chemicalbook.com

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The Roche researchers studied the binding of nutlin-2 to HDM2 (Vassilev et al., 2004). Nutlin-2 (which differs from nutlin-3 by having bromophenyl rings in place of the chlorophenyl rings of nutlin-3, plus two side-chain differences) mimics the interaction of the p53 N-terminal domain with HDM2, by interacting with the binding sites in the HDM2 binding pocket for the three critical amino acids of the p53 domain.

Since the discovery of the nutlins, Roche researchers and their collaborators have been studying nutlin-3 in various cell culture and preclinical animal models, as a monotherapy and in combination therapies (Shangary and Wang, 2009; Cheok et al., 2011). These studies showed that nutlin-3 potently induced apoptosis in cell lines derived from such hematologic cancers as acute myeloid leukemia (AML), acute lymphoblastoid leukemia (ALL), MM, and B-cell chronic lymphocytic leukemia (B-CLL). These hematologic tumors (as well as pediatric cancers) are particularly likely targets for treatment with nutlin-3 and other HDM2/p53 PPI disrupting agents, since they exhibit a high percentage of unmutated TP53 at diagnosis.

Nutlin-3 also induced tumor shrinkage in several mouse xenograft models bearing tumors derived from human cancers with wild-type TP53. The drug showed little toxicity *in vivo*. This contrasts with studies involving genetic deletion of the MDM2 gene in mice, which shows embryonic lethality (Cheok et al., 2011). Researchers have found functions for MDM2 other than inhibiting p53 and hypothesize that deletion of MDM2, which leads to complete loss of these functions, is toxic, while inhibition of MDM2's p53 inhibition

function is not. Moreover, although nutlin-3 is toxic to primary tumor cells in culture, it is not toxic to normal cells and tissues.

Preclinical studies of nutlin-3 in combination with other oncology drugs in various tumor models have also been reported (Cheok et al., 2011). Nutlin-3 synergizes with a wide variety of such agents, including cytotoxic agents such as vinblastine, doxorubicin, fludarabine, gemcitabine, cyclin-dependent kinase inhibitors that target the cell cycle (e.g. roscovitine), the aurora kinase inhibitor VX-680, and radiation. Nutlin-3 also synergizes with tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL), and bortezomib (Secchiero et al., 2011). These studies support the development of nutlins in cancer, either as single agents or in combination therapies. They led to the entry of the nutlin-3 analog RG7112 into Phase I clinical trials in hematologic malignancies and advanced solid tumors.

MI-219

Another inhibitor of the HDM2/p53 PPI is MI-219, which is in advanced preclinical studies. MI-219 was discovered in the laboratory of Shaomeng Wang at the University of Michigan (Shangary et al., 2008; Shangary and Wang, 2009). Ascenta Therapeutics (Malvern, PA, USA), which has designated MI-219 as AT-219, is developing this and related compounds licensed from the University of Michigan. In June 2010, Ascenta licensed its HDM2/p53 program to Sanofi, granting that company an exclusive worldwide license to develop, manufacture, and commercialize compounds issued from the program. In return, Ascenta received an upfront payment, and may receive development, regulatory, and commercial milestone payments with a potential value of $398m. In addition, Ascenta is eligible to receive royalties on any sales of products deriving from this program.

Starting with the X-ray crystallography-based structural information on the HDM2/p53 PPI discussed earlier, the Michigan researchers used structure-based drug design and computational modeling to discover nonpeptidic inhibitors of this interaction. Because the indole ring of the tryptophan 23 residue of p53 is buried deeply inside the hydrophobic cavity of the HDM2 hotspot, and its NH group forms a hydrogen bond with a backbone carbonyl in this cavity, the researchers reasoned that tryptophan 23 appears to be the most critical amino acid residue for binding of p53 to HDM2. Therefore, the researchers performed a search for chemical moieties that can mimic the interaction of tryptophan 23 with HDM2. They found that in addition to an indole

ring such as is found in tryptophan, oxindole can mimic the side chain of tryptophan 23 for interaction with HDM2.

The researchers then did a search to identify natural products that contain an oxindole moiety. They identified two compounds that contained a core structure that could be used as the starting point for the design of a new class of HDM2 inhibitors. The oxindole moiety in this core structure could mimic the tryptophan 23 side chain, and the spiropyrrolidine ring in the core structure could be used as a scaffold to add groups that could mimic the side chains of phenylalanine 19 and leucine 26.

From this starting point, the researchers designed compounds via a docking program, ultimately arriving at a lead compound that they tested in an MDM2 binding assay. The compound bound with micromolar affinity and was then optimized to give a compound that had an inhibition constant of 8.5μM. Further rounds of optimization for HDM2 binding based on computer modeling and the binding assay, followed by optimization for pharmacological properties, yielded MI-219. MI-219 binds to HDM2 with an inhibition constant (Ki) of 5nM, compared with 36nM for nutlin-3. MI-219 is designed to mimic not only phenylalanine 19, tryptophan 23, and leucine 26 in the p53 binding site for HDM2, but also a fourth residue, leucine 22, which based on mutational analysis (including alanine scanning) also appears to play an important role in the HDM2/p53 PPI (Shangary and Wang, 2009).

MI-219 has good pharmacological properties, including 55% oral bioavailability in mice (Shangary and Wang, 2009). It shows a high degree of specificity for MDM2; for example, it showed over 10,000-fold selectivity for MDM2 over MDM2's closely related homolog MDMX. In cancer cells with wild-type p53, MI-219 induced accumulation of p53. The drug also inhibited growth of cancer cell lines with wild-type p53 with submicromolar IC_{50} values, but it exhibited 20- to 50-fold weaker inhibition in cancer cell lines with mutated p53.

MI-219 and related compounds have been tested in various mouse models of cancer (Cheok et al., 2011). The MI-219 analog MI-319 was shown to be effective against a model of follicular lymphoma as well as a model of systemic follicular small-cleaved-cell lymphoma. MI-219 analogs have also been tested and have demonstrated activity in combination therapies with etoposide, doxorubicin, and cisplatin in mouse models of

lung cancer, rhabdomyosarcoma, and pancreatic cancer. These agents were found to have minimal toxic effects in normal cells.

JNJ-26854165

Johnson & Johnson's JNJ-26854165, a novel tryptamine derivative in Phase I clinical trials in advanced or refractory solid tumors, was an agent with an unknown mechanism of action that had been deemed to target the HDM2/p53 PPI (Cheok et al., 2011). However, researchers at the MD Anderson Cancer Center (Houston, TX) investigated the mechanism of action of JNJ-26854165 in 2010, and it appears to induce apoptosis in malignant cells via a different mechanism that does not involve HDM2 (Kojima et al., 2010). Instead, in tumor cells with wild-type p53, it appears to work via accelerating the proteasomal degradation of p21 and to antagonize the p53-mediated transcriptional induction of p21. Interestingly, JNJ-26854165 also induces apoptosis in tumor cells with mutant p53 via inducing delay in the S-phase of mitosis and upregulation of expression of the transcription factor E2F1 (a key mediator of an important pathway that controls cellular proliferation). This results in apoptosis, preferentially of S-phase cells.

In addition to the role of E2F1 in the induction of apoptosis by JNJ-26854165, there is evidence that nutlin-3-induced apoptosis may require E2F1-mediated transcriptional activity (Kitagawa et al., 2008). Although nutlin-3 inhibits growth in most tumor cells that express wild-type p53, it only induces apoptosis in subsets of these tumor cells. From various studies, there is evidence that the apoptotic activity of nutlin-3 correlates with transcriptional activity of E2F1, and depletion (via siRNA) of E2F1 and its downstream transcriptional target p73 (a p53 homolog) suppresses nutlin-3-induced apoptosis. Such results suggest that tumor cells that express wild-type p53 and have high E2F1 transcriptional activity may be good targets for nutlin-3 therapy.

MDM4/MDMX and therapy with HDM2/p53 inhibitors

MDM4 (also known as MDMX; the version in human cells is sometimes called HDM4 or HDMX), a structural homolog of MDM2, may also be expressed in normal and tumor cells (Cheok et al., 2011). This biomolecule regulates p53 stability and activity, in part by forming a heterocomplex with HDM2. As with HDM2, MDM4 interacts with the N-terminal domain of the transactivation domain of p53.

MDM4 can interfere with HDM2-targeting therapies in tumors that express high levels of MDM4 (e.g. including many pediatric cancers, as well as some adult tumors that overexpress MDM4) (Cheok et al.,

2011). In that case, inhibition of both HDM2 and MDM4 may be necessary. Small-molecule compounds that target MDM4 and that bind to this protein with a low micromolar binding constant have recently been discovered (Reed et al., 2010). If researchers can further optimize these compounds, they may be used in combination with HDM2/p53 inhibitors such as nutlins to target cancers that express wild-type p53 and overexpress MDM4.

Inhibitors of other E3 ubiquitin ligases via disruption of PPIs

Work on discovering compounds that disrupt interactions between other E3 ubiquitin ligases and their substrates is in an early stage (Cohen and Tcherpakov, 2010).

Researchers identified a small-molecule inhibitor of Cdc4, which is the yeast ortholog of the mammalian CRL Fbw7 (F box and WD repeat domain-containing 7) (Orlicky et al, 2008). (Human Fbw7 is a tumor suppressor (Welcker and Clurman, 2008).) Via X-ray crystallography, the researchers found that the compound binds to Cdc4 at a site that is remote from the substrate-binding site. Binding of the inhibitor induces a conformational change that affects the substrate-binding pocket, thus impeding its ability to recognize the substrate protein. The compound thus acts as an allosteric inhibitor. This study raises the possibility that other CRLs that contain structural features (known as WD40 domains) similar to the allosteric binding site of this Cdc4 inhibitor may also be targeted by allosteric inhibitors.

Via a chemical genetics screen in yeast to identify small-molecule enhancers of rapamycin (which works via the TOR pathway), researchers serendipitously discovered a small-molecule inhibitor of the SCFMet30 ligase, which was designated as SMER3 (Aghajan et al., 2010; Cohen and Tcherpakov, 2010). (The connection between SCFMet30 ligase and the TOR pathway remains unexplained.) SCFMet30 is a yeast E3 ligase that regulates the cell cycle via ubiquitinylation of the transcription factor Met4. The mammalian homolog of SCFMet30 is SCF3Keap1, and the mammalian homolog of Met4 is Nrf2 (Kaiser et al., 2006). SMER3 was found to bind directly to SCFMet30, and to inhibit its binding to the SCF core complex (which was mentioned earlier) *in vivo* (Aghajan et al., 2010). Further details of SMER3's mechanism of action (i.e. whether it functions as a direct or an allosteric PPI modulator) are unknown.

These two examples of the discovery of small-molecule modulators of yeast E3 ubiquitin ligases represent basic research and have no direct clinical applications. However, they demonstrate that an expansion of the field of discovery of E3 modulators beyond HDM2/p53 is feasible.

Conclusions

The discovery and development of small-molecule compounds that target the ubiquitin system is a young field but a potentially important one. This potential importance is demonstrated not only by studies on the basic biology of the ubiquitin system but also by the success of the first drug that targets this system, the proteasome inhibitor bortezomib (Millennium's Velcade). Despite its nonspecificity and limited field of application, bortezomib has achieved blockbuster status, with annual sales of $1.4bn as of 2010 (Cohen and Tcherpakov, 2010).

Since the launch of bortezomib onto the market, drug discovery researchers have been developing strategies to target several levels of the UPS, including the proteasome, E2s, and E3s. Among these compounds, as discussed earlier, are second-generation proteasome inhibitors that appear to have a more favorable adverse-effect profile than bortezomib. Drugs in development that target the ubiquitinylation cascade itself include the neddylation inhibitor MLN4924, which indirectly targets the class of E3s known as CRLs, and the E2 inhibitor CC0651, an allosteric effector that indirectly targets a subset of CRLs. Neither of these compounds is a PPI modulator.

The PPI modulators in development both target the interaction between HDM2 and p53. They are the nutlin-3 analog RG7112 (Roche; in Phase I clinical trials in hematologic malignancies and advanced solid tumors), and MI-219 and its analogs (Ascenta/Sanofi; in advanced preclinical trials). Another compound, JNJ-26854165 (Johnson & Johnson; in Phase I clinical trials in advanced or refractory solid tumors) was until recently thought to be a possible HDM2/p53 inhibitor but now appears to work via a different mechanism of action. It appears, however, to target the p53 pathway and to have similar effects on tumors to HDM2/p53 inhibitors like the nutlins and MI-219.

As expected for such a young field, the number of compounds in development is small. Moreover, PPI modulators represent only a subset of these compounds and so far target only one PPI. Nevertheless, given

the small number of PPI inhibitors currently in the clinic, the two HDM2/p53 inhibitors are significant. Moreover, given the large number and specificity of E3 ubiquitin ligases and their important role in intracellular pathways, there is a large field of possibility for discovery of novel PPI modulators that target these biomolecules and their interactions with their substrates.

Chapter 6 Small molecules targeting protein-protein interactions that control apoptosis

Summary

- Apoptosis is the major and best-studied pathway of programmed cell death in all multicellular animals. It is an ATP-dependent, programmed, and orderly process of cellular suicide. Apoptosis is essential for normal embryonic development, and for maintaining normal cellular homeostasis in adults, as well as for response to infectious agents. It is dysregulated in several major diseases. Cancer is the major focus of researchers seeking to develop drugs that modulate apoptotic pathways, since apoptosis is blocked in perhaps all cancers. This is a significant factor in uncontrolled cellular proliferation in cancer.
- The central pathways of apoptosis are controlled by a complex system of pro- and antiapoptotic Bcl-2 family members, which act to ensure that apoptosis is only triggered when it is appropriate. Bcl-2 family member interactions that control apoptosis are PPIs. Thus it has been difficult to discover agents that affect the central pathways of apoptosis and which are capable of being taken into the clinic.
- There are now two Bcl-2 family PPI disrupting agents in clinical trials: Abbott/Genentech's navitoclax and Gemin X/Cephalon/Teva's obatoclax.
- Navitoclax (ABT-263) is the result of the fragment-based drug discovery methodology known as SAR by NMR, which led to a Bcl-2 inhibitor designated as ABT-737. The researchers later optimized ABT-737 to produce a second-generation compound, navitoclax, which has improved physicochemical and pharmacological properties and is orally available.
- Abbott and Genentech are codeveloping navitoclax. It is in Phase I and Phase II clinical trials in various cancers, including as a combination with targeted therapies such as Rituxan (rituximab) and Tarceva (erlotinib), as well as with cytotoxic chemotherapies and as a single agent.
- Obatoclax (GX15-070) was discovered by Gemin X, which in 2011 was acquired by Cephalon; later in 2011, Cephalon was acquired by Teva. The Gemin X researchers discovered the drug by screening natural product libraries, and optimizing the resulting lead compound.

- Obatoclax is a potent inhibitor of Mcl-1. Mcl-1 is overexpressed in several types of cancer and undergoes rapid steady-state turnover by the 26S proteasome complex. As a result, treatment of malignancies with proteasome inhibitors like bortezomib results in accumulation of Mcl-1. This can result in interference with the therapeutic response to bortezomib. This suggests that a good Mcl-1 inhibitor such as obatoclax might be useful in these cases in which Mcl-1 is important in blocking apoptosis.

Introduction

Apoptosis is the major and best-studied pathway of programmed cell death and is conserved in all multicellular animals (Danial and Korsmeyer, 2004; Wyllie, 2010). It is an ATP-dependent, programmed, and orderly process of cellular suicide. In embryonic development, apoptosis is essential for the successful formation of complex multicellular tissues and organs.

Apoptosis also is important in adult organisms, especially in long-lived mammals such as humans, to maintain normal cellular homeostasis. For example, in humans and other mammals, skin and hair cells, as well as cells of the intestinal lining, are renewed via a continuous cycle of cell division and apoptosis. As a result, these tissues renew themselves, as new cells replace old ones. In the immune system, the vast majority of the precursors of T and B cells that are generated throughout an organism's life die via apoptosis. This is part of the process by which lymphocytes that can react to non-self antigens are selected for, and those that react to self-antigens are selected against.

Apoptosis is also important in response to infectious agents. Viral infection may trigger apoptosis in infected cells, thus halting viral replication and preventing spread of the virus to uninfected cells. Cytotoxic T lymphocytes that recognize virus-infected cells kill the cells that they recognize via inducing apoptosis. Once again, this prevents spread of the virus, and helps control and eliminate the infection.

Dysregulation of apoptosis is a key factor in several major human diseases. Insufficiency in apoptosis is involved in the pathogenesis of cancer and autoimmunity, and accelerated apoptosis is involved in the pathogenesis of stroke and neurodegenerative diseases, immunodeficiency, and infertility. Cancer is the major focus of most researchers and companies seeking to develop drugs that modulate apoptotic pathways.

Apoptotic pathways

Central to apoptotic pathways are two families of proteins, the caspases and the Bcl-2 family (Danial and Korsmeyer, 2004; Wyllie, 2010). Caspases (or cysteine aspartyl proteases) are a class of serine proteases that function in apoptosis. They also have a specific four amino acid motif (including aspartate at positions 1 and 4) at their target site. The B-cell lymphoma-2 (Bcl-2) family, which is named for the first member to be discovered, the Bcl-2 oncogene, is a diverse family of apoptosis regulatory proteins, which as discussed later include proapoptotic and antiapoptotic members. In general, caspases form a cascade that ultimately results in cell death. Bcl-2 family proteins control this process, either halting the processes that result in apoptotic cell death, or allowing these processes to go forward.

The intrinsic pathway of apoptosis

The central pathways of apoptosis include an intrinsic and an extrinsic pathway. The intrinsic pathway is triggered by such factors as cellular stress (for example, deprivation of growth factors needed for survival, or treatment with various drugs or ionizing radiation), p53-mediated apoptotic signals triggered by DNA damage, virus infection, hypoxia, and energy deprivation. The intrinsic pathway is also modulated by other signal transduction pathways, such as “oncogene overdrive” in which the Myc oncogene may trigger apoptosis instead of hyperproliferation and malignant transformation (Boone et al., 2011), the Akt/PTEN pathway (which when dysregulated is a factor in several types of cancer), and the UPS pathway for degradation of unwanted or defective intracellular proteins, which was discussed in Chapter 5. As was discussed in that chapter, several UPS pathway inhibitors can trigger apoptosis in susceptible tumor cells. This chapter focuses on means to directly modulate apoptotic pathways, not on modulation of such pathways as the p53, Myc, and Akt/PTEN pathways, and the proteasomal degradation pathway.

Triggers of the intrinsic pathway operate mainly via modulating members of the Bcl-2 family (Danial and Korsmeyer, 2004; Wyllie, 2010). Bcl-2 family proteins possess homologous domains that can enter into PPIs among the family members. Antiapoptotic members of the Bcl-2 family, such as Bcl-2, Bcl-xL, and Mcl-1, possess four conserved domains, called BH1, BH2, BH3, and BH4. BH 1, 2, and 4 define a hydrophobic groove within the molecule, and BH3 is an eight to12 amino acid domain that binds within that groove. These antiapoptotic proteins localize to the mitochondria, where they specifically bind and sequester proapoptotic

multidomain Bcl-2 family members, such as Bak and Bax. These multidomain proapoptotic proteins contain three of the domains of the antiapoptotic proteins – BH1, BH2, and BH3, but not BH4. The remaining members of the Bcl-2 family (e.g. Bid, Bad, Bim, Bmf) possess only the BH3 domain, and are all proapoptotic. Their precise mode of action is still disputed. However, they bind with high affinity, via their BH3 domain, to the hydrophobic groove of the antiapoptotic family members. This is thought to release the BH-1/2/3 apoptotic proteins from their complexes with the antiapoptotic proteins.

Once released from these complexes, the proapoptotic BH-1/2/3 proteins can form homo-oligomers, which create a wide diameter pore through the outer mitochondrial membrane. This results in the escape of critical molecules from the intermembranous space of the mitochondrion to the microenvironment immediately next to the mitochondrion. Among these molecules are cytochrome c and deoxyadenosine triphosphate (dATP). (Cytochrome c is best known for its function in oxidative phosphorylation, the principal means by which the cell derives its energy.)

Once released into the perimitochondrial cytoplasm, cytochrome c complexes with a protein known as the apoptotic protease activating factor-1 (Apaf-1) and caspase 9 to form a complex called the apoptosome (Danial and Korsmeyer, 2004; Wyllie, 2010). Caspases are synthesized as inactive zymogens, which must be activated by specific proteolysis events to display enzyme activity. In the apoptosome in the presence of adenosine triphosphate (ATP) or deoxyadenosine triphosphate (dATP), caspase 9 undergoes autocatalytic activation. Caspase 9 is known as an initiator caspase, since once activated it can activate other caspases. In particular, it activates “effector caspases” or “executioner caspases,” such as caspases 3 and 7. These go on to catalyze a series of proteolytic events that result in all the hallmarks of apoptosis, such as DNA fragmentation, blebbing, and eventual formation of apoptotic bodies. Apoptotic bodies are cell fragments that phagocytic cells can engulf and quickly remove before the intracellular contents can cause damage to surrounding cells. This contrasts with the situation in another mode of cell death, necrosis. In necrosis (traumatic cell death, which can be caused by toxins, anoxia, infections, trauma, etc.), cellular contents leak out from dead and dying cells, resulting in damage to surrounding cells and the triggering of an immune response. (However, researchers have recently defined a more controlled type of necrosis known as “programmed necrosis” (Chan FK and Baehrecke, 2012). This form of necrosis utilizes some similar

pathways to those involved in the extrinsic pathway of apoptosis, discussed in the next section. The biology of cell death thus still includes significant unknowns.)

The extrinsic pathway of apoptosis

The extrinsic apoptotic pathway is triggered by a class of cell-surface receptors of the tumor necrosis factor receptor (TNFR) family and their corresponding tumor necrosis factor (TNF) family ligands (Danial and Korsmeyer, 2004). These receptor/ligand pairs include TNFR/TNF-α (tumor necrosis factor-alpha), Fas/FasL (Fas ligand), and TRAIL receptor/TRAIL (TRAIL = TNF-related apoptosis inducing ligand). In addition to its apoptotic function, TNF-α also functions in inflammation. The Fas/FasL system is involved in killing by cytotoxic T cells and regulation of the immune system. TRAIL is of great interest to cancer biologists and oncology drug developers, since it has been found to selectively induce apoptosis in cancer cells, independent of p53, which is usually inactivated in human cancers. However, the physiological role of TRAIL is not well understood. Nonetheless, there is evidence that TRAIL may be involved in various inflammatory processes such as those involving dendritic cells and natural killer cells (Zaba et al., 2010).

Binding of a specific ligand to TNFR family receptors on the cell surface results in clustering of the receptors. The intracellular domains of the receptor complexes then bind to multiple copies of death adaptor proteins, such as Fas-associated death domain protein (FADD). These in turn bind to caspase 8, an initiator caspase that, once bound, is autocatalytically activated. Caspase 8 can then activate effector caspases such as caspase 3 and 7, leading to apoptosis. Caspase 8 also can initiate the intrinsic program of apoptosis via cleavage of the inactive p22 form of Bid. This results in formation of an active form of Bid, which triggers the intrinsic pathway of apoptosis via mitochondria membrane pore formation by complexing of proapoptotic Bcl-2 family proteins like Bak and Bax.

Alternatively, engagement of TNFR receptors can lead to survival and cell proliferation rather than apoptosis. This is in part regulated via complexing of the intracellular domains of the clustered receptors with prosurvival adaptor proteins, which, for example, couple the TNFR1 receptor to the proinflammatory nuclear factor kappa B (NF-κB) pathway. This pathway not only is prosurvival but also induces cell proliferation and/or production of proinflammatory cytokines.

Abbott/Genentech's Bcl-2 inhibitor ABT-263 (navitoclax)

The interest of cancer researchers and of pharmaceutical and biotechnology companies in developing Bcl-2 inhibitors stems from the role of Bcl-2 overexpression, with resulting blockage of apoptosis, in numerous cancers (Adams and Cory, 2007). In human follicular lymphoma, Bcl-2 is activated via a chromosomal translocation. This leads to increased survival of tumor cells via a shift in the balance between pro- and antiapoptotic Bcl-2 family members toward blockage of apoptosis.

This led to the concept, now widely accepted by cancer researchers, that impairment of apoptosis is a critical step in tumor progression. Researchers hypothesize that apoptosis normally eliminates most cells in which oncogenic mutations have resulted in the dysregulation of cell-cycle control. Impaired apoptosis would promote survival of not only such preneoplastic cells but also those bearing other types of oncogenic and pro-metastatic mutations. Researchers have also found that overexpression of antiapoptotic members of the Bcl-2 family promotes resistance to cytotoxic chemotherapy. For example, in the National Cancer Institute (NCI) panel of 60 diverse cancer cell lines, the expression level of the antiapoptotic Bcl-xL highly correlates with resistance to cytotoxic agents (Adams and Cory, 2007).

As discussed earlier in this chapter, antiapoptotic BH-1/2/3/4 Bcl-2 family members such as Bcl-2 and Bcl-xL exert their antiapoptotic activity via sequestering proapoptotic multidomain (BH-1/2/3) Bcl-2 family members such as Bak and Bax. Proapoptotic BH3-only proteins such as Bid, Bad, and Bim can bind with high affinity, via their BH3 domain, to the hydrophobic groove of the antiapoptotic family members. This can release the BH-1/2/3 apoptotic proteins from their complexes with the antiapoptotic proteins; the BH-1/2/3 proteins are then free to complex with each other and initiate the intrinsic pathway of apoptosis. However, if BH-1/2/3/4 antiapoptotic proteins are overexpressed and are thus in excess, as in many cancers, release of BH-1/2/3 apoptotic proteins is impaired, and survival of the cancer cells is favored.

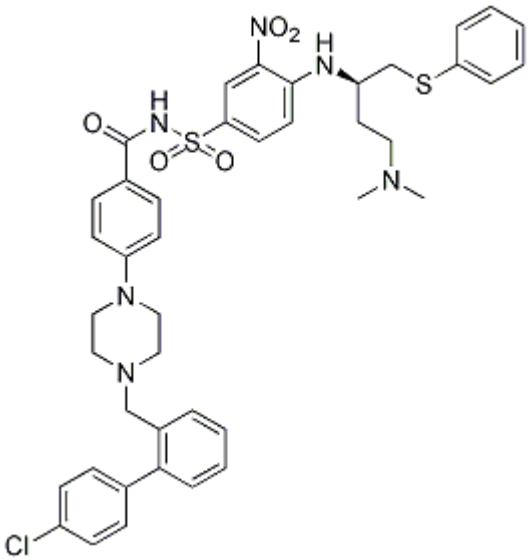
All the interactions between the Bcl-2 family members are PPIs and have thus been deemed at one time to be undruggable. Drug discovery researchers wishing to disrupt interactions between Bcl-2 or Bcl-xL and BH-1/2/3 apoptotic proteins therefore must ether develop alternative types of therapeutics such as antisense or siRNA compounds, or must solve the hard problem of discovering small-molecule drugs that specifically disrupt the relevant PPIs. Genta (Berkeley Heights, NJ) has been attempting to develop an antisense agent

against Bcl-2 since 2002. However, its Phase III drug candidate oblimersen (Genasense) showed disappointing results in melanoma, and the company discontinued the drug in May 2011.

In Chapter 2 of this report, we discussed the work of Stephen Fesik (then at Abbott) and his colleagues that led to the discovery and development of small-molecule Bcl-2 inhibitors that target the PPIs formed by Bcl-2 and related proteins. As discussed in that chapter, these researchers developed the fragment-based drug discovery (FBDD) method known as SAR by NMR. Together with Idun (San Diego, CA) and with academic collaborators, they then applied this technology to the discovery of inhibitors of Bcl-2 family proteins (Oltersdorf et al., 2005). (Pfizer acquired Idun in 2005, and in 2010 Conatus Pharmaceuticals (San Diego, CA) acquired Idun from Pfizer.)

The researchers used SAR by NMR to screen a chemical library to identify fragment-sized small molecules that bound to the hydrophobic BH3-binding groove of Bcl-xL. They identified two fragments that bound, respectively, to two distinct but proximal subsites within the groove. The subsites are the same as those that are occupied, respectively, by aspartic acid 83 and leucine 78 of a peptide derived from the BH3 domain of the BH-1/2/3 proapoptotic protein Bak. There are two of the three residues most critical for the affinity of Bak for Bcl-xL. Via linkage and optimization, including modification to reduce binding to human serum albumin, the researchers used these two fragments as the starting point to create a compound known as ABT-737 (Figure 13). ABT-737 bound with subnanomolar affinity to Bcl-XL, Bcl-2, and Bcl-w, but only with micromolar affinity to less homologous proteins such Bcl-B and Mcl-1. Because of the binding of compounds such as ABT-737 to the BH3 binding site of antiapoptotic B-1234 proteins, these small molecules are known as “BH3 mimetics.”

Figure 13: Chemical structure of ABT-737



Source: www.selleckchem.com

INFORMA

The researchers found that ABT-737 did not in itself initiate release of cytochrome c from mitochondria, but antagonized protection by Bcl-2 and Bcl-xL against mitochondrial cytochrome release. ABT-737 disrupted intracellular PPIs between Bcl-xL and the proapoptotic Bcl-xS.

ABT-737 also displayed synergism with various cytotoxic agents and radiation in killing a variety of tumor cell lines *in vitro*, reducing the dose of the cytotoxic compounds or radiation needed to achieve 50% killing of these cells. ABT-737 also displayed potent single-agent killing activity against cell lines derived from lymphoid malignancies and small-cell lung cancer (SCLC), but not cell lines derived from a variety of other solid tumors.

In mouse xenograft models, ABT-737 caused complete regression of two types of established SCLC tumors, which did not grow back in a high percentage of the mice over the course of the study. (ABT-737 is not orally bioavailable in mice, so was administered intraperitoneally). *In vivo* ABT-737 treatment resulted in increases in activated caspase-3 in SCLC xenografts (as determined by antibody staining), which suggests that tumor regression was due to apoptotic cell death. Normal tissues showed no increase in caspase-3 activation.

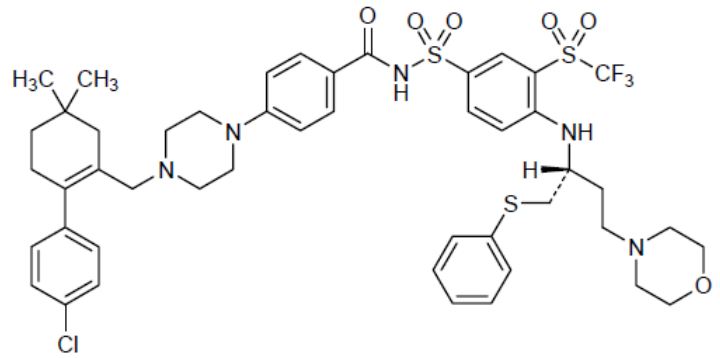
ABT-737 administration did not result in significant weight loss in mice treated for 21 days, although reductions in platelets and lymphocytes were seen.

This study suggests that the Bcl-2 family PPI inhibitor ABT-737 might be useful for the treatment of lymphoma and SCLC as a monotherapy, and of a wide variety of cancers in combination with cytotoxic agents or radiation.

In 2008, Fesik and his Abbott colleagues published a report on the optimization of ABT-737, which aimed to improve its physiochemical and pharmaceutical properties (Tse et al., 2008). ABT-737 is not orally bioavailable, and its low solubility makes formulation for intravenous delivery challenging. In the *in vitro* xenograft studies, ABT-737 had to be administered continuously, which would not be practical for human administration. These issues would render ABT-737 a poor prospect for clinical development.

In the 2008 paper, Fesik and his colleagues reported the further optimization of ABT-737 to produce a second-generation, orally available BH3 mimetic, ABT-263 (Figure 14). Optimization of ABT-737, a molecule that is larger (over 800 Daltons) than the 500 Dalton Lipinski molecular weight cutoff, required a balance between target affinity, cellular potency, and oral absorption. Interestingly, the desired balance was eventually achieved not by reducing the high molecular weight but instead by studying variations in three portions of the molecule that affected charge balance, metabolism, and oral absorption. The resulting compound, ABT-263 (Figure 14), maintains the subnanomolar affinity for Bcl-2, Bcl-xL, and Bcl-w, but binds weakly to Mcl-1, as in the case of ABT-737. Remarkably, the molecular weight of the optimized ABT-263 is even greater than that of its predecessor, ABT-737.

Figure 14: Chemical structure of ABT-263 (navitoclax)



Source: WHO Drug Information Vol. 24, No. 2, 2010 **INFORMA**

The researchers performed several studies to rigorously show that ABT-737-induced cytotoxicity was due to disruption of PPIs between antiapoptotic BH-1/2/3/4 proteins such as Bcl-2 and antiapoptotic members of the Bcl-2 family (Tse et al., 2008). ABT-737 reversed protection from apoptotic cell death due to overexpression of Bcl-2 or Bcl-xL in the interleukin-3 (IL-3)-dependent prolymphocytic FL5.12 murine cell line. Coimmunoprecipitation studies in the same cells and in other cell lines showed that ABT-737 induced a dose-dependent decrease in Bim:Bcl-xL interactions. Several other studies also indicated that ABT-737 induced cytotoxicity via upregulating the intrinsic pathway of apoptosis via disruption of PPIs between Bcl-2 or Bcl-xL and such proapoptotic proteins as Bim and Bax in several cell lines, including human tumor cell lines.

Other studies showed that oral administration of ABT-737 caused regression of SCLC and acute lymphoblastic leukemia (ALL) tumors in mouse xenograft models. Other *in vivo* studies demonstrated that oral administration of ABT-737 synergized with such anticancer agents as rituximab and bortezomib, as well as with the R-CHOP (rituximab, cyclophosphamide, hydroxydaunorubicin, Oncovin, prednisone) combination.

ABT-263 (as with its predecessor ABT-737) was found to induce a rapid but reversible thrombocytopenia in dogs (Tse et al., 2008). This adverse effect is mechanism-based (i.e. it is due to induction of the intrinsic

pathway of apoptosis in platelets via inhibition of antiapoptotic Bcl-2 family members). However, although platelet levels fall after the first dosing of the drug, subsequent daily dosing to achieve a sustained plasma level of ABT-263 does not significantly reduce platelet levels below that observed after administration of the first dose. Doses of ABT-263 that are several-fold above the efficacious level of the drug did not reduce platelet count below the level that would constitute clinical grade 3 thrombocytopenia. This suggests that there is a therapeutic window for the drug, which would allow safe dosing in humans. However, this issue must of course be tested in Phase I clinical trials.

Abbott has designated ABT-263 as navitoclax, which it is now codeveloping with Genentech. (Conatus, the successor company of Idun, has rights to milestone and royalty payments as navitoclax progresses through clinical trials.) Navitoclax is in Phase I and Phase II clinical trials in various cancers, including combination therapies with such Genentech drugs as Rituxan (rituximab) and Tarceva (erlotinib), as well as with cytotoxic chemotherapies and as a single agent.

In a recent report of a Phase I study of navitoclax as a monotherapy in patients with chronic lymphocytic leukemia (CLL), navitoclax showed substantial activity (Roberts et al., 2012). Of 26 patients treated with navitoclax, nine achieved a partial response and seven maintained stable disease for over six months. Median progression-free survival was 25 months. The main dose-limiting toxicity was thrombocytopenia due to Bcl-xL inhibition; this toxicity was dose-related. On the basis of the Phase I study, the researchers determined an optimal dose for Phase II studies and concluded that further studies of navitoclax as a monotherapy and in combination for treatment of CLL are warranted.

Obatoclax, a pan-Bcl-2 inhibitor that inhibits MCl-1

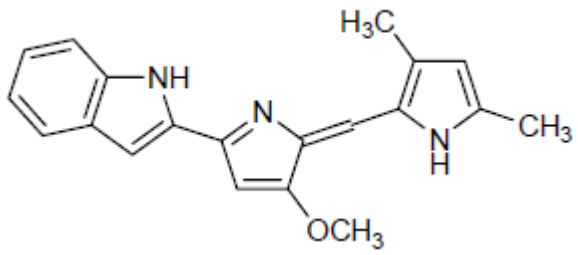
As discussed in the previous section, ABT-737 and ABT-263 (navitoclax) bind with high affinity to the antiapoptotic Bcl-2 family proteins Bcl-XL, Bcl-2, and Bcl-w, but are weak inhibitors of Mcl-1 (induced myeloid leukemia cell differentiation protein). However, Mcl-1 is overexpressed in several types of cancer, for example in the majority of prostate cancers (Dash et al., 2010). Moreover, Mcl-1, unlike other pro-survival members of the Bcl-2 family, undergoes rapid steady-state turnover by the 26S proteasome complex. As a result, treatment of malignancies with proteasome inhibitors like bortezomib results in accumulation of Mcl-1.

This can result in interference with the therapeutic response to bortezomib in malignancies treated with this drug (Nguyen et al., 2007). Therefore, PPI inhibitor drugs that target Mcl-1 (which exerts its antiapoptotic activity by complexing with Bak at the mitochondrion) would be useful.

Such a drug, obatoclax (GX15-070), was discovered by Gemin X (Malvern, PA) (Shore and Viallet, 2005; Nguyen et al., 2007). Gemin X was acquired by Cephalon (Frazer, PA) in March 2011, and Cephalon in turn was acquired by Teva in October 2011.

The Gemin X researchers discovered the drug by using a high-throughput protein-protein interaction assay to screen natural product libraries. This resulted in the identification of a lead compound that falls within the polypyrrole class of molecules. Optimization of this lead compound resulted in GX15-070 (obatoclax) (Shore and Viallet, 2005). The structure of this compound is shown in Figure 15.

Figure 15: Chemical structure of obatoclax



Source: WHO Drug Information, Vol. 19, No. 4, 2005 **INFORMA**

In a study of the ability of several small molecules to inhibit binding of a BH3 peptide to recombinant versions of the cytosolic domains (i.e. the proteins with the C-terminal domain that crosses the mitochondrial membrane omitted) of the pro-survival Bcl-2 family proteins Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and BCL-B in a fluorescence polarization assay, obatoclax was found to inhibit the interaction for all these proteins at micromolar levels (Zhai et al., 2006). Under the conditions of this aqueous-based assay, ABT-737 exhibited more potent (nanomolar) inhibition of Bcl-2, Bcl-xL, and Bcl-w than did obatoclax; however, ABT-737 was not effective in inhibiting the other three proteins, including Mcl-1.

Interpretation of the results of the above study is complicated by the hydrophobicity of obatoclax. In aqueous-based assays, obatoclax is nearly insoluble, with a log P (logarithm of the water/octanol partition coefficient,

a measure of hydrophobicity) of greater then 4. Thus the inhibition constants determined for obatoclax were deemed to be inaccurate. A binding constant determined for obatoclax *in silico* (as determined by Gemin X researchers and their academic collaborators (Nguyen et al., 2007)) with the BH3-binding groove of Bcl-2 was approximately 220nM, compared with the experimentally-determined inhibition constant of 7.01μM.

Because the hydrophobicity of obatoclax would be expected to render it compatible with biological membranes, the Gemin X researchers and their academic collaborators studied the disruption of constitutive Mcl-1/BAK PPIs in intact mitochondria. Studies in isolated mitochondria and in lymphoma cells indicated that obatoclax significantly and potently inhibited these PPIs. Other cell culture studies indicated that obatoclax inhibited the Mcl-1 apoptotic pathway by disrupting the Mcl-1/BAK and the Mcl-1/BAX PPIs, and that obatoclax overcame Mcl-1-mediated resistance to apoptosis in lymphoma cells. Obatoclax also overcame resistance to apoptosis in cultured melanoma cells treated with bortezomib (Nguyen et al., 2007).

Because of its poor solubility, obatoclax cannot be an oral drug. However, when formulated for intravenous administration, obatoclax showed single-agent antitumor activity in mouse xenograft models bearing several different types of human carcinomas (Nguyen et al., 2007).

Cephalon has been studying obatoclax (which is also designated as CEP-41601, and earlier as GX15-070) in Phase II clinical trials for first-line treatment of SCLC in combination with the cytotoxic agents carboplatin and etoposide. It is also in Phase II clinical trials for the treatment of leukemia, lymphoma, myelofibrosis, and mastocytosis (ClinicalTrials.gov, 2011).

Conclusions

The central pathways of apoptosis are controlled by a complex system of pro- and antiapoptotic Bcl-2 family members, which act to ensure that apoptosis is only triggered when it is appropriate. In perhaps all cancers, apoptosis is blocked, which is a significant factor in tumor cell proliferation and metastasis. Therefore, researchers would like to discover and develop agents that unblock apoptotic pathways in cancer cells. However, since Bcl-2 family member interactions that control apoptosis are PPIs, it has been difficult to discover agents that affect the central pathways of apoptosis and that are capable of being taken into the clinic.

Nevertheless, there are now two Bcl-2 family PPI disrupting agents (BH3 mimetics) in clinical trials – Abbott/Genentech's navitoclax and Gemin X/Cephalon/Teva's obatoclax.

The optimization of ABT-737 – a poor candidate for clinical development due to its unfavorable physicochemical properties – which resulted in a second-generation, orally available BH3 mimetic, ABT-263, which is now in clinical trials, illustrates an important point for the development of small-molecule PPI modulators. Many medicinal chemists and others remain skeptical about the ability of researchers to develop small-molecule drugs that target protein-protein interactions, which have satisfactory physicochemical properties for entry into and advancement through clinical trials (Keller et al., 2006). In the case of ABT-737, fragment-based drug design produced a compound with potent PPI inhibitory activity, but which had unfavorable physicochemical properties (including a high molecular weight) for development of a drug, thus seemingly reinforcing the skeptics' doubts. Nevertheless, medicinal chemists at Abbott were able to optimize it (notably without diminishing molecular weight) to produce an improved compound, ABT-263, which has advanced into Phase II clinical trials.

Chapter 7 Stapled peptides for targeting protein-protein interactions

Summary

- In parallel with the discovery and development of small-molecule PPI modulators, and of second-generation technologies for the discovery of such compounds, other researchers have been designing peptides that mimic protein domains that are involved in PPIs. This involves mimicking not only the amino acid sequence of these domains, but also their secondary structure. It has been proposed that "stapled peptides," which contain a small loop rigidifying the peptide conformation, will able to penetrate cells, be resistant to degradation by proteolytic enzymes, and have favorable pharmacological properties.
- The private, venture capital-funded discovery-stage biotechnology company Aileron Therapeutics (Cambridge, MA) was founded in 2005 to develop and commercialize the stapled peptide technology invented by Gregory Verdine (Harvard University) and his colleagues
- Aileron has been building a pipeline of stapled-peptide agents targeting Bcl-2 family members that control apoptosis, the HDM2/p53 PPI (for p53 reactivation), and the notch signal transduction pathway. It also has an HIV1 capsid formation inhibitor (a potential anti-HIV/AIDS drug).
- Roche has entered into a collaboration with Aileron to discover, develop and commercialize stapled peptide agents against undisclosed targets in cancer, virology, inflammation, metabolism and neurology. Aileron announced in November 2011 that it had achieved a key milestone related to *in vivo* proof of concept in one of the programs in which it is collaborating with Roche, thus triggering a milestone payment. The Roche/Aileron agreement constitutes an important validation of Aileron's stapled peptide technology and pipeline.
- Stapled-peptide technology for the development of PPI modulators represents a promising alternative to the development of small-molecule PPI modulators. However, stapled peptide agents are – unlike numerous small-molecule PPI modulators – not orally available. Moreover, as yet not one of Aileron's

agents has entered the clinic. Thus the stapled peptide field awaits the achievement of proof of concept in human studies.

Introduction

So far, this report has focused on the discovery and development of small-molecule PPI modulators. Chapter 2 discussed the technologies and strategies that form the basis for the discovery of small-molecule PPI modulators that have so far entered into the clinic and in a few cases reached the market. The discovery of these compounds has been difficult and has been on a “one compound at a time” basis. Chapters 3–6 focused on case studies of the “one compound at a time” discovery and development of these compounds.

Chapter 2 also included discussions of novel, second-generation enabling technologies that are designed to allow researchers and companies to more readily and consistently discover small-molecule PPI modulators, and thus to make the development of these compounds a commercial proposition on a consistent basis. These technologies form the basis for the technology platforms of Forma Therapeutics and Ensemble Therapeutics, and several large pharmaceutical companies have collaborations with these two companies aimed at the development of small-molecule PPI modulators. However, these are early days in the applications of these technologies, and the fruits of these efforts have not yet entered the clinic.

Meanwhile, at the same time as researchers have been developing these second-generation technologies for the discovery of small-molecule PPI modulators, other researchers have been taking a quite different approach. Instead of aiming to discover small-molecule PPI modulators, these researchers have been designing peptides that mimic protein domains that are involved in PPIs. Designing such peptide mimics involves mimicking not only the amino acid sequence of these domains, but also their secondary structure. If these mimics are to serve as drugs, they must be able to penetrate cells, be resistant to degradation by proteolytic enzymes, and must have favorable pharmacological properties. At least several of these mimetics, known as “stapled peptides,” which in some cases have reached the preclinical stage, appear to fulfill all these criteria.

Aileron Therapeutics and stapled peptide technology

The discovery-stage biotechnology company Aileron Therapeutics, a private biotech firm funded by venture capital, was founded in 2005 to develop and commercialize its proprietary stapled peptide technology. The company's scientific founders include Gregory Verdine (Department of Chemistry and Chemical Biology, Harvard University), Loren Walensky (Dana-Farber Cancer Institute), and the late Stanley J. Korsmeyer (Dana-Farber Cancer Institute). Korsmeyer was a pioneer in the study of the Bcl-2 family and its role in apoptosis and the biology of cancer; this was the focus of Chapter 6 of this report.

Verdine and his colleagues are the inventors of the stapled peptide technology, which has been exclusively licensed to Aileron (Walensky et al., 2010, Schafmeister et al., 2000, Kim et al., 2011). The initial application of stapled peptide technology was to the Bcl-2 family PPI system, which as discussed in Chapter 6 controls apoptosis. As discussed in Chapter 6, Bid is one of the BH3-only proapoptotic members of the Bcl-2 family. It appears to exert its proapoptotic activity by binding to the hydrophobic groove of antiapoptotic BH-1/2/3/4 proteins such as Bcl-2 and Bcl-xL. This releases proapoptotic BH-1/2/3 proteins from their complexes with the antiapoptotic proteins, enabling them to initiate the intrinsic pathway of apoptosis. Beginning in the mid-1990s, Korsemeyer, in collaboration with Verdine and Walensky, wished to develop a Bid mimetic peptide.

The BH3 domain of Bid, which triggers apoptosis by the above mechanism, has an α-helical structure. However, when researchers synthesize a peptide with the amino acid sequence of the Bid BH3 domain, it loses its α-helical structure, is ineffective in disrupting PPIs between Bcl-2 or Bcl-xL and BH-1/2/3 proapoptotic proteins, and is subject to degradation by serum and cellular proteases. Moreover, such peptides cannot penetrate cells.

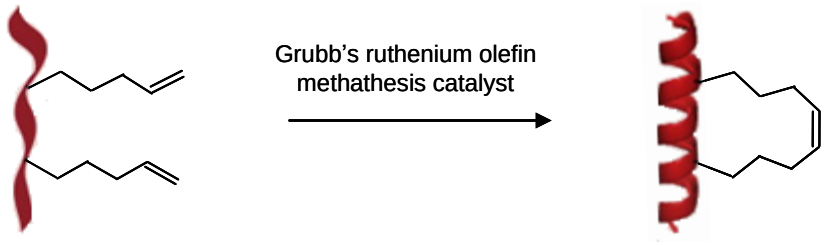
Verdine and his colleagues applied their stapled peptide technology to this problem, to produce a stable α-helical form of the peptide. To construct stapled peptides, researchers synthesized peptides with the amino acid sequence of the domain of interest (in this case, the Bid BH3 domain), but incorporating two appropriately spaced non-natural amino acids bearing olefin side chains (Walensky et al., 2004; Kim et al., 2011).

To design these peptides, the researchers use the three-dimensional structures of the target protein and the interacting partner domain that will provide the sequence of the stapled peptide. (These structures may be obtained, for example, from the Protein Data Bank, as was discussed in Chapter 2.) The researchers then use molecular visualization software, to analyze the binding interface between these two biomolecules. They determine if the partner binding domain is α-helical. If it is (which is often the case), the researchers select amino acid sequences that are not directly involved in target recognition as candidates for substitution with non-natural amino acids. They then synthesize peptides, each of which contains two olefin-bearing amino acid residues 3, 4, or 7 amino acid residues apart. These modified peptides are designed to place the reactive olefin residues on the same face of the α-helix.

The researchers then form the "staple" (i.e. the bridge between the two non-natural amino acids) by use of ruthenium-mediated ring-closing olefin metathesis. (The use of olefin metathesis in the synthesis of macrocyclic compounds and libraries was mentioned in Chapter 2). The "staple" in a stapled peptide forms a macrocyclic ring with the amino acid residues it encompasses. The range of bridge lengths of 3, 4, or 7 amino acid residues is used to identify the optimal stapled peptide with respect to affinity for its target and other properties relevant to serving as a chemical tool or a drug candidate.

The construction of a stapled peptide is illustrated in Figure 16.

Figure 16: Construction of a stapled peptide



Source: Haberman Associates

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Peptide stapling results in stabilization of the α-helical form of such peptides as the Bid BH3 domain and other peptides derived from domains that are α-helical in their natural proteins. Such peptides can also be cell-penetrant. Hydrocarbon staples promote effective cellular uptake via endocytic vesicle trafficking (Kim et al., 2011). Cellular penetration by a stapled peptide is also dependent on the net charge of the molecule; those with net positive charges show better cellular uptake.

Stapled peptides exhibit activity against their partner proteins in their cellular context, whether intracellular or extracellular. They are also resistant to proteases, apparently because the helical structure results in burying the amide backbone of the peptide, making the amide groups inaccessible to proteases. Stapled peptides can also have *in vivo* activity against their targets, which suggests that they may be useful as drugs.

In the original application of the stapled peptide technology, Walensky, Verdine, and Korsmeyer and their colleagues constructed stapled peptide Bid BH3 mimetics that they called “stabilized alpha-helix of Bcl-2 domains” (SAHBs) (Walensky et al., 2004). SAHBs were α-helical, protease-resistant, and penetrated cells. Biophysical studies showed that one of the SAHBs that was studied further, SAHBA, caused the same structural changes in Bcl-xL as did Bid.

SAHBA was found to induce cytochrome c release from isolated mouse liver mitochondria. SAHBA, as well as Bid, failed to induce cytochrome c release from isolated mitochondria that lacked the proapoptotic BH-1/2/3 proteins Bak and Bax. Thus SAHBA-mediated cytochrome c release from mitochondria in this *in vitro* system is dependent on the intrinsic pathway of apoptosis.

SAHBA also was found to penetrate Jurkat cells (a human T-cell leukemia cell line) and to induce apoptosis in them. Overexpression of Bcl-2 in Jurkat cells made them resistant to induction of apoptosis by SAHBA. However, use of higher doses of SAHBA resulted in induction of apoptosis. This is consistent with SAHBA functioning to overcome Bcl-2-mediated inhibition of apoptosis. SAHBA also was found to induce apoptosis in other types of leukemic cells.

Intravenous administration of SAHBA consistently caused tumor regression in mouse xenograft models of human leukemia, as well as improved survival (median survival five days for control animals, and 11 days for SAHBA-treated animals). SAHBA showed no overt toxicity in normal tissues.

The studies designed to create a Bid mimetic using stapled peptide technology provided a proof of principle for the technology *in vitro* and in animal models, and served as a starting point for Aileron's efforts to build a pipeline of preclinical candidates based on stapled peptides.

Targeting the notch pathway using a stapled peptide

In 2009, Verdine and his colleagues published a report on the design and the use of a stapled peptide to target the notch signal transcription pathway (Moellering et al., 2009).

The evolutionarily conserved notch pathway regulates various aspects of cell-cell communication, cellular differentiation, cell proliferation, and cellular survival or death. For example, the notch pathway is essential for the development of the nervous and hematopoietic systems. Deregulation of the notch pathway is involved in various cancers, including cancers of the lung, ovary and pancreas, and in T-cell acute lymphoblastic leukemia (T-ALL), which is a cancer of immature T cells.

Notch is a cell-membrane receptor, as are its ligands (i.e. members of the Delta, Serrate, and Lag-2 family). The notch pathway becomes activated when the extracellular domain of notch binds to a notch ligand on the surface of an adjacent cell. Binding of an appropriate ligand to the extracellular domain of notch triggers sequential proteolytic cleavage of the notch intracellular domain, first by an ADAM (A Disintegrin And Metalloproteinase) family metalloprotease such as TACE (tumor necrosis factor alpha converting enzyme, also known as ADAM17) and then by a γ-secretase complex (γ-secretase is also involved in the amyloid pathway that has been implicated in Alzheimer's disease).

The free intracellular domain of notch, known as the intracellular domain of NOTCH1 (ICN1), translocates to the nucleus, and docks with the DNA-bound transcription factor CSL (CBF1, Suppressor of Hairless, Lag-1). The interaction between CSL and ICN1 creates a long shallow groove along the interface of the two proteins, which serves as a docking site for coactivator proteins of the mastermind-like (MAML) family such as MAML1. The resulting trimolecular complex initiates specific transcription of notch-dependent target genes.

As with other key signal transduction pathways, the notch pathway is centered on PPIs, especially the crucial ternary transcription factor complex CSL/ICN1/MAML1. The discovery of small-molecule drugs that modulate this pathway had thus been considered infeasible. Verdine and his colleagues therefore wished to apply their

stapled peptide technology to discovering agents to modulate this pathway, in particular the key transcription factor complex.

Previous studies by other researchers found that a dominant-negative fragment of MAML1, designated as dnMAML1 (consisting of amino acid residues 13–74) antagonized notch signaling and cell proliferation when expressed in T-ALL cells (Moellering et al., 2009). X-ray diffraction studies showed that the dnMAML1 polypeptide formed an α-helix, which docks with the elongated groove formed by the ICN1/CSL complex. This suggested that it might be possible to design a stapled peptide based on a portion of the sequence of dnMAML1. Such a stapled peptide might inhibit binding of MAML1 to the ICN1/CSL complex, thus blocking transcription of downstream notch-dependent target genes.

The researchers therefore synthesized a set of six short candidate peptides, which together encompassed the entire contact surface of dnMAML1 with the ICN1/CSL complex (Moellering et al., 2009). Functional studies with these peptides led the researchers to select the 15-amino-acid stapled peptide SAHM1. The researchers showed that stapling conferred a marked helical character (94% helical) to SAMH1, compared with its non-stapled counterpart.

SAMH1 was found to be cell-penetrant. Biochemical studies showed that SAMH1 bound to the ICN1/CSL complex competitively with MAML1. Cell culture studies in the KOPT-K1 human T-ALL cell line with a notch-regulated fluorescent reporter gene showed that SAMH1 specifically repressed transcription of notch target genes, in a dose-dependent manner. Gene expression analysis in KOPT-K1 and HPB-ALL human T-cell leukemia cells showed that SAMH1 specifically repressed transcription of notch pathway downstream target genes.

The researchers also found that SAMH1 markedly reduced proliferation of several T-ALL cell lines *in vitro* but was ineffective against leukemia cell lines that do not depend on the notch pathway for cell proliferation. In sensitive T-ALL cell lines, SAMH1 treatment also triggered apoptosis.

In a mouse model of T-ALL, twice-daily intraperitoneal injection of SAMH1 resulted in a significant dose-dependent regression of leukemia, compared with vehicle-treated mice. Studies of mononuclear cells from

SAMH1- and vehicle-treated mice confirmed that SAMH1 treatment resulted in a significant decrease in notch target gene transcription.

These studies showed that SAMH1 is a direct transcriptional antagonist of the notch pathway. This suggests that SAHM1 can be used to determine the role of this pathway in normal physiology and development, and in disease processes. SAMH1 also provides a starting point for drug development, especially in notch-driven cancers such as T-ALL.

Aileron's therapeutic programs

Aileron says that it has built a broad and growing pipeline of stapled peptides that address many therapeutic areas, and that it is now advancing such drug candidates through preclinical development. The company's strategy is to target PPIs that serve as control points for disease pathways, using target-specific, cell-penetrant stapled peptides. Via internal programs and in collaboration with Roche, Aileron is initially focusing on cancer, infectious diseases, metabolic diseases, and immune/inflammatory diseases. (The Roche collaboration is discussed later in this section.)

Table 6 lists stapled peptide drug candidates that were constructed by Aileron researchers and their academic collaborators, based on publications that support Aileron's therapeutic programs, and which are listed on the company's website. The Bcl-2 inhibitor SAHBA and the notch transcription factor complex inhibitor SAHM1, which are discussed earlier in this chapter, are included in Table 6.

Table 6: Aileron's therapeutic pipeline

Stapled peptide	Target	Reference
SAHBA (Bid BH3 mimetic)	Bcl-2, Bcl-xL, etc.; intrinsic apoptosis pathway	Walensky et al., 2004
SAHM1 (MAML1 binding domain mimetic)	ICN1/CSL transcription factor complex; notch pathway	Moellering et al., 2009
SAH-p53-8 (p53 transactivation domain mimetic)	HDM2/p53 PPI; p53 reactivation	Bernal et al., 2007
NYAD-1 (stapled form of the 12-mer peptide CAI, identified by phage-display.)	HIV1 capsid formation inhibitor; potential anti-HIV1 drug	Zhang et al, 2008
Mcl-1 SAHB (Mcl-1 BH3 mimetic)	Mcl-1-controlled apoptosis	Stewart et al., 2010

Source: Haberman Associates, based on information from Aileron Therapeutics website INFORMA

Other stapled peptides included in Table 6 include agents designed to target the HDM2/p53 PPI, and Mcl-1-controlled apoptosis. As discussed in Chapter 5 and Chapter 6, respectively, these targets are also the subjects of small-molecule PPI modulator development, as is Bcl-2. The need for specific Mcl-1 modulators because of the significant differences of Mcl-1 from Bcl-2 and Bcl-xL was discussed in Chapter 6 with respect to small-molecule modulators; this situation applies to stapled peptides as well.

As discussed in Chapter 5, MDM4/MDMX can interfere with p53 reactivation by such small-molecule HDM2/p53 modulators as the nutlins. Interestingly, Aileron's stapled peptide HDM2/p53 modulator SAH-p53-8 has a 25-fold greater binding preference for HDMX (also known as MDM4) compared with HDM2 (Bernal et al., 2010). MDM4 thus does not interfere with SAH-p53-8-mediated reactivation of p53, and SAH-p53-8 restores the ability of nutlin-3a to reactivate p53 in cells with elevated expression of MDM4.

Two of Aileron's programs listed in Table 6 do not involve targets that have as yet been addressed by small-molecule drug candidates. One is the notch pathway modulator that was discussed previously. The other is an inhibitor of HIV1 virion assembly, which is a potential anti-HIV1 drug.

The Aileron/Roche collaboration

Roche's involvement with Aileron began in 2009. In June of that year, Aileron closed a $40m Series D venture capital round, which included the participation of four big pharma venture funds: SR One (GlaxoSmithKline's independent corporate healthcare venture fund), Novartis Venture Fund, Lilly Ventures, and Roche Venture Fund. In August 2010, Roche entered into a collaboration with Aileron that constitutes the first industry collaboration for stapled peptide technology. Under the agreement, Aileron and Roche were to discover, develop, and commercialize stapled peptide agents against up to five undisclosed targets in cancer, virology, inflammation, metabolism, and neurology. Aileron was to be responsible for the majority of discovery and preclinical development, while Roche was to be responsible for clinical development and commercialization. Under the agreement, Aileron was to receive $25m in technology access fees and R&D support and would also be eligible for up to $1.1bn in milestones, as well as royalties for any commercialized products.

In November 2011, Roche expanded its collaboration with Aileron to also include inflammatory diseases. The financial details of this expanded program were undisclosed. Also in November 2011, Aileron announced that it had achieved a key milestone related to *in vivo* proof of concept in one of the programs in which it is collaborating with Roche. This triggered a substantial milestone payment.

According to Aileron CEO Joseph Yanchik, until the signing of the Roche agreement, all discussions about stapled peptides as a drug platform came out of Aileron's presentations and out of publications by Aileron researchers and their academic collaborators (Weintraub, 2011). The investment and participation of a large pharmaceutical company in stapled peptide research adds an additional level of validation to the stapled peptide approach. Nevertheless, all of Aileron's stapled peptide agents are in the research or preclinical stage. Thus there is not yet any clinical proof of concept that a stapled peptide drug may be safe and efficacious in humans.

Conclusions

The stapled-peptide technology, developed by Gregory Verdine and his colleagues and exclusively licensed to Aileron Therapeutics, represents a promising alternative to the development of small-molecule PPI

modulators as outlined in Chapters 2–6 of this report. The design of stapled peptides that modulate a PPI of interest is much more straightforward than the discovery or design of a corresponding small-molecule PPI modulator. Moreover, the discovery/research phase of stapled peptide PPI modulator development – from concept to preclinical testing – should have a shorter timeline than that of a small-molecule PPI modulator.

Aileron researchers and their academic collaborators have accumulated considerable evidence that stapled peptide agents can have many desirable properties of drugs – including lack of toxicity, cell penetration, high affinity for their targets, good pharmacokinetics and pharmacodynamics, and efficacy in animal models. However, stapled peptide agents are – unlike numerous small-molecule PPI modulators – not orally available.

Aileron has been building a pipeline of preclinical-stage stapled-peptide agents and has demonstrated proof of principle in animal models that several of its agents are efficacious and have little or no toxicity. The company's approach has received validation via the Roche collaboration and investment. However, as yet not one of Aileron's agents has entered the clinic. Thus the stapled peptide field awaits the achievement of proof of concept in human studies.

Chapter 8 Outlook for protein-protein interaction modulators

Summary

- The discovery and development of protein-protein interaction modulators has been difficult, due to the structure of protein-interacting interfaces, the lack of natural ligands for PPIs to serve as starting points for drug design, and the unsuitability of proprietary pharmaceutical company or commercially available chemical libraries for use in HTS campaigns to identify compounds that modulate PPIs.
- Despite the difficulty of discovering and developing PPI modulatory drugs, developing PPI modulators is becoming of increasing strategic importance to the pharmaceutical and biotechnology industry. Companies need to address hitherto “undruggable” targets, especially PPIs, to reverse the low productivity of pharmaceutical R&D. It is estimated that all known small-molecule drugs address only 2% of human proteins. Most of the remaining proteins are designated “intractable.” Most of the proteins that are critically involved in disease pathways lie within the category of intractable targets, and most of these targets are PPIs.
- Despite the difficulties in discovering small-molecule PPI modulators that are capable of being taken into clinical trials, researchers and companies have done so in several cases. Central to these successes has been the determination of “hotspots” in protein-protein interfaces. By targeting hotspots, several compounds that directly modulate PPIs have been discovered. However, this has been on a sporadic “one compound at a time” basis, which has usually been slow and laborious.
- Among these examples is one compound that has reached the market – eltrombopag (Ligand/GSK’s Promacta/Revolade) – as well as several others currently in clinical trials. In addition to these direct PPI modulators, there are also allosteric chemokine receptor modulators, including two marketed drugs – maraviroc (Pfizer’s Selzentry/Celsentri) and plerixafor (Genzyme’s Mozobil) – and several others now in clinical trials.

- The sporadic, “one compound at a time” approach cannot meet the strategic needs of the pharmaceutical/biotechnology industry to expand the numbers of targets that can be addressed by developable drugs. Because of the difficulty in making PPI modulator development a commercial proposition on a consistent basis, all the pioneering companies in this field (e.g. Genentech, Roche, Sunesis, and Abbott) abandoned or cut back on their PPI modulator programs, and several of the pioneering industry researchers in the field have moved to academic positions.
- Researchers have been developing second-generation technologies designed to enable the development of small-molecule and peptide PPI modulators on a more consistent basis. These include computational solvent (CS) mapping, diversity-oriented synthesis of chemical libraries, Ensemble Therapeutics’ proprietary DNA-programmed chemistry (DPC) technology for synthesis of libraries of macrocyclic compounds, and stapled-peptide technology.
- Responding to these new technologies, pharmaceutical and biotechnology companies are moving back into the PPI modulator field. In addition to companies developing compounds discovered via older technologies that are already in clinical trials, companies active in PPI modulator discovery include Ensemble, Forma, Aileron, Bristol-Myers Squibb, Pfizer, Novartis, Ajinomoto, Ligand, Ascenta, Sanofi, Cephalon/Teva, and Roche. In several cases, these companies have been collaborating with one another, and/or with academic groups.
- Whether the new suite of enabling technologies for PPI modulator discovery and development will enable this area to be commercially successful, or to meet the strategic needs of the industry for expanding the universe of targets for drug discovery and developing drugs that address them, remains an open question. All of the compounds that have been discovered using these technologies are in the research and preclinical stage. It will be necessary for some of these compounds to enter the clinic, achieve proof of concept, and reach the market before the value of the novel technologies can be assessed. Nevertheless, the PPI modulator field in 2012 is an exciting area that is gaining increasing interest and investment by leading pharmaceutical and biotechnology companies.

Discovery and development of PPI modulators has been difficult

Drug discovery researchers have long considered protein-protein interactions (PPIs) to be the prototypical "undruggable" targets, and those who have nevertheless attempted to discover small-molecule PPI modulators have found that indeed it has been very difficult.

As discussed in Chapter 1, there is a set of theoretical reasons for why discovering small-molecule PPI modulators has been so challenging. The contact surfaces involved in protein-protein interfaces are large and usually flat, compared with the smaller contact surfaces and definitive binding pockets that characterize interactions between proteins and small molecules. Moreover, unlike the enzymes and receptors that have been good targets for small-molecule drug development, PPIs do not have natural small-molecule ligands that can be used as starting points for the design of drugs. Moreover, high-throughput screening (HTS) with proprietary pharmaceutical company or commercially available chemical libraries does not usually identify compounds that modulate PPIs. Most contact surfaces between interacting proteins also involve amino-acid residues that are not contiguous in polypeptide chains. In the great majority of cases, this makes the design of peptidomimetics based on polypeptide sequences in the protein-protein interface unfeasible.

Targeting PPIs is becoming increasingly important for the success of the pharmaceutical industry

As discussed in Chapter 1, despite the difficulty of discovering and developing PPI modulatory drugs, developing PPI modulators is becoming of increasing strategic importance to the pharmaceutical and biotechnology industry. Pharmaceutical companies have been experiencing low R&D productivity in recent years, which, combined with the loss of patent protection of blockbuster drugs, has been threatening company revenues.

Several recent analyses single out target selection as the key factor that is limiting the productivity of pharmaceutical R&D in the current era (Bunnage, 2011; Stockwell, 2011, reviewed in Lowe, 2011). As discussed in Chapter 1, these commentators cite studies indicating that drug discovery researchers are running out of "druggable" targets that have not already been addressed by drugs that are on the market.

Therefore, researchers will need to focus on novel targets (identified by genomics studies) that have been neglected by drug discoverers, targets that are involved in such recently opened-up research areas as epigenetic regulation and the ubiquitin-proteasome system, and targets that have been deemed to be "undruggable."

Notable among the "undruggable" or intractable targets are the PPIs. PPIs are involved in nearly every disease process, including the biological pathways involved in many types of cancer, neurodegenerative diseases, immune/inflammatory diseases, metabolic diseases (e.g. type 2 diabetes and obesity), and many others. Transcription factors and many other signaling proteins work via interacting with other proteins in PPIs. Since the human interactome (the whole set of PPIs in human cells) is estimated to include between 130,000 and 650,000 PPIs, even if only a small fraction were relevant to disease and could be addressed by drugs, PPIs would still constitute an enormous are of unaddressed target opportunity.

As discussed in Chapter 1, expanding the universe of potential targets for drug discovery will be essential for drug discovery researchers to identify quality targets (i.e. targets that are disease-relevant, that are addressable by drugs, and that can yield safe drugs that can provide significant improvements in efficacy over current standards of care). In particular, if the pharmaceutical industry wishes to expand its universe of potential targets and thus make progress in R&D, it needs to develop suites of tools to address PPIs and other hitherto "undruggable" targets.

In a recent blog published by Nature, Stockwell notes that all known small-molecule drugs address only 2% of human proteins (Stockwell, 2012). Most of the remaining proteins are designated as "challenging," "intractable," or "undruggable" targets. However, most proteins that are critically involved in disease pathways lie within the category of intractable targets. This suggests that it may not be possible to discover and develop disease-modifying drugs for the diseases controlled by these proteins. As Stockwell notes, most of the intractable proteins function by interacting with other proteins, and thus fall in the PPI class.

As shown by the above recent publications, industry experts are recognizing the emerging strategic importance of PPIs as drug targets, and the important, and perhaps crucial role of PPI drugs in reversing the productivity crisis in pharmaceutical R&D.

Researchers have discovered PPI modulators and moved them into the clinic

Despite the difficulties in discovering small-molecule PPI modulators that are capable of being taken into human trials, researchers and companies have done so in several cases. As described in Chapter 2, the discovery of these compounds was made possible by a set of tools developed by the pioneers in the field, beginning in the late 1980s and 1990s. These include such methods as X-ray diffraction, alanine scanning mutagenesis, and fragment-based drug discovery, coupled with medicinal chemistry. Central to the structural biology and mutagenesis components of this tool kit has been the determination of “hotspots” in protein-protein interfaces. In the large, generally flat binding interfaces between interacting proteins, only a small subset of amino acid residues contributes most of the free energy of binding. These amino acid residues define a hot spot, which generally forms a pocket that interacts with a complementary hotspot on the surface of the interacting face of the other protein. These interacting hotspots are potential targets for drug discovery.

Based on these tools and on the targeting of hotspots, several compounds that modulate PPIs have been discovered, on a “one compound at a time” basis, which has usually been slow and laborious. In some cases, these compounds have been successfully optimized by medicinal chemists and taken into the clinic. Table 7 lists clinical stage compounds that are direct modulators of PPIs.

Table 7: Clinical-stage PPI modulators

Target	Compound	Comments
Thrombopoietin (TPO) receptor	Eltrombopag (SB-497115-GR) (Ligand/GSK's Promacta/Revolade)	Agonist; approved for idiopathic thrombocytopenic purpura (ITP); in Phase III trials in hepatitis C and in Phase II trials in oncology
Thrombopoietin (TPO) receptor	GSK2285921 (formerly LGD-4665); Ligand/GSK	Agonist; Phase II, oncology
Lymphocyte function-associated antigen (LFA-1)/ intercellular adhesion molecule-1 (ICAM-1) PPI	SARcode's SAR1118 (originally developed by Sunesis)	Antagonist; Phase III, dry eye syndrome (LFA-1/ICAM-1 is a target in inflammatory conditions, including dry eye)
HDM2/p53 PPI	Roche's RG7112, an analog of nutlin-3	Antagonist; Phase I, hematologic malignancies and advanced solid tumors
Bcl-2 and Bcl-xL	Abbott/Genentech's navitoclax (ABT-263)	Antagonist of antiapoptotic Bcl-2 family PPIs; Phase I and Phase II clinical trials in various cancers
Bcl-2 family proteins, including Mcl-1	Gemin X/Cephalon/Teva's obatoclax (CEP-41601; formerly GX15-070)	Phase II, small-cell lung cancer, leukemia, lymphoma, myelofibrosis, and mastocytosis

Source: Haberman Associates

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Among the compounds listed in Table 7 is the marketed drug eltrombopag (Ligand/GSK's Promacta/Revolade), which was described more fully in Chapter 3. This thrombopoietin (TPO) receptor agonist is approved for the treatment of the rare disease idiopathic thrombocytopenic purpura (ITP), and it is also in Phase III clinical trials to treat low platelet count in patients with cirrhosis of the liver due to hepatitis C and in Phase II trials in oncology. A follow-on compound to eltrombopag, Ligand/GSK's GSK2285921, is in Phase II clinical trials in oncology. Table 7 also lists four other compounds in clinical trials in such conditions as inflammation (specifically, dry eye syndrome), and cancer.

In addition to the direct PPI modulators listed in Table 7, there are also small-molecule chemokine receptor antagonists in development, which appear to be allosteric modulators. Leading examples of such compounds are listed in Table 4. These chemokine receptor antagonists were discovered mainly via fairly standard medicinal chemistry and high-throughput screening methods, unlike the direct PPI modulators listed in Table 7. Among the chemokine receptor antagonists listed in Table 4 are two marketed compounds – the HIV entry inhibitor maraviroc (Pfizer's Selzentry/Celsentri) and plerixafor (Genzyme's Mozobil), which is used in combination with G-CSF to mobilize hematopoietic stem cells to the peripheral blood for autologous transplantation in patients with non-Hodgkin lymphoma and multiple myeloma.

Among the other chemokine receptor antagonists listed in Table 4 is ChemoCentrix/ GSK's Traficet-EN (GSK'786), which is in Phase III clinical trials in Crohn's disease. The other agents listed in the table are in Phase II clinical trials.

As discussed in Chapter 3, although the discovery of small-molecule allosteric chemokine antagonists is easier in terms of chemistry than is the discovery of direct small-molecule PPI modulators, the development of chemokine receptor antagonists has been difficult due to the complex biology of the diseases that are addressed by these compounds. Poorly predictive animal models of these diseases, as well as target redundancy, have been particularly important issues. As a result of these difficulties, most chemokine receptor antagonists that have been entered into clinical trials have failed.

Nevertheless, there are promising chemokine receptor antagonists in clinical trials, especially Traficet-EN (GSK'786). Therefore, at least a few of these compounds should reach the market within the next several years.

In addition to the direct small-molecule PPI modulators in clinical trials listed in Table 7, there are also numerous preclinical direct small-molecule PPI modulators that have been discovered. At least one of these compounds is in advanced preclinical studies. This is the HDM2/p53 PPI inhibitor MI-219 (which had been licensed by Ascenta from the University of Michigan, and designated as AT-219; this and related compounds were subsequently licensed to Sanofi). This and other preclinical small-molecule PPI modulators – or optimized versions of these compounds – may be expected to reach the clinic in the next several years.

A key point that was discussed in Chapter 6 was the need for careful optimization of small-molecule PPI modulators to obtain compounds with physicochemical properties that would enable them to be good clinical candidates. This issue came up in conjunction with the development of Abbott's Bcl-2 antagonist ABT-737, which was a poor candidate for clinical development due to its unfavorable physicochemical properties. Abbott researchers optimized this compound to produce the second-generation, orally available BH3 mimetic, ABT-263, which is now in clinical trials. Many medicinal chemists and others remain skeptical about the ability of researchers to develop small-molecule drugs that target protein-protein interactions with high specificity and affinity, and which also have satisfactory physicochemical properties for entry into and advancement through clinical trials (Keller et al., 2006). This is especially the case with compounds discovered via fragment-based drug discovery, which often have high molecular weights. ABT-737 is such a compound. Nevertheless, medicinal chemists at Abbott were able to optimize it – notably without recourse to molecular weight reduction – to produce an improved compound, ABT-263, which has advanced into Phase II clinical trials.

Roche also optimized its HDM2/p53 PPI inhibitor nutlin-3 to produce its clinical candidate RG7112, which has improved potency and pharmacological properties (Andreeff et al., 2010).

These two examples indicate that, at least in some cases, it is possible to optimize PPI modulators discovered via the methods discussed earlier in this section to produce oral compounds with favorable pharmacological properties, if due attention is given to the issue of optimization. However, in the case of the poorly soluble obatoclax, it was not possible to optimize the compound to produce an oral drug. Nevertheless, researchers were able to produce a formulation of obatoclax for intravenous administration, which has been taken into the clinic (Chapter 6).

In conclusion, despite the "undruggable" designation previously assigned to PPIs by medicinal chemists and others, dedicated groups of researchers have been able to discover direct and allosteric small-molecule PPI modulators that could be taken into the clinic. In a few cases, such compounds have emerged onto the market. Given the current pipeline of PPI modulators, we expect additional compounds to reach the market over the next several years.

Nevertheless, the time, effort, and cost required to discover, optimize, and develop small-molecule PPI modulators, via the methodology developed by the pioneers in the field beginning in the late 1980s and 1990s, are considerable. Consequently, the development of compounds via these methods is on a sporadic, "one compound at a time" basis. Such an approach cannot meet the strategic needs of the pharmaceutical/biotechnology industry to greatly improve R&D productivity by expanding the numbers of targets that can be addressed by developable drugs. As noted in Chapter 2, because of the difficulty in making PPI modulator development a commercial proposition on a consistent basis, all the pioneering companies in this field (e.g. Genentech, Roche, Sunesis, and Abbott) abandoned or cut back on their PPI modulator programs, and several of the pioneering industry researchers in the field have moved to academic positions.

New technologies are enabling the development of small-molecule and peptide PPI modulators

As discussed in Chapter 2, the discovery of compounds to modulate PPIs to date has represented a "premature technology," in other words a field of biomedical science in which, despite the exciting potential of the field, consistent practicable therapeutic applications are in the future, due to difficult technological hurdles (Haberman, 2009). To advance from the "premature technology" stage, the field of small-molecule PPI modulators requires the development of a set of enabling technologies to drive the field up the technology development curve and render the discovery and development of these drugs routine or at least fairly commonplace. The pioneers of the PPI modulator field, as discussed earlier in this chapter and in Chapter 2, did develop a set of enabling technologies for the discovery of small-molecule PPI modulators. However, these represent first-generation technologies that have not been robust enough to enable the regular discovery and development of these drugs.

Chapter 2, however, includes a discussion of a set of second-generation technologies that are aimed at moving the field of small-molecule PPI modulator discovery and development up the technology curve. Among these technologies is computational solvent (CS) mapping, which is a virtual analog of such fragment-based drug discovery (FBDD) methods as SAR by NMR. CS mapping was developed by Sandor Vajda and his colleagues at Boston University. Using only the X-ray diffraction structures of unliganded

proteins (which can usually be obtained from published databases), researchers can use CS mapping to identify potential binding pockets for fragment-sized small molecules in PPI interfaces, and to combine these determinations with a set of rules to account for the flexibility of nearby side chains, which would be necessary for the binding site to accommodate a drug-size inhibitor. Without the need for such laborious and time-consuming methods as site-directed mutagenesis/alanine scanning, SAR by NMR, tethering, or other types of FBDD, researchers can use this virtual methodology to determine if a protein or PPI is druggable, and to guide the design of small-molecule PPI modulators.

Chapter 2 also includes discussion of novel chemistry technologies. These include diversity-oriented synthesis, which represents a set of methodologies for building chemical libraries that cover larger portions of chemical space than do libraries derived from standard combinatorial chemistry, and which include complex “natural product-like” compounds that are likely to have biological activity. In recent years, novel DOS strategies have been devised and implemented, notably Stuart Schreiber’s modular “Build/Couple/Pair” (B/C/P) strategy, which enables chemists to synthesize diverse libraries in a small a number of steps. As described in Chapter 2, researchers have screened libraries derived via DOS synthesis strategies, and have found compounds that have biological activity, including the ability to modulate PPIs and other difficult targets.

Another important development in chemical science and technology that is expected to enable researchers to discover novel PPI modulators is the new focus on macrocyclic compounds, both in academia and in industry. The focus on macrocycles is in part driven by the rise of DOS and especially of the B/P/C strategy, since many of the compounds synthesized via DOS are macrocycles. Many natural products, including numerous drugs, are macrocycles, and several of these drugs modulate PPIs.

Natural products, including macrocyclic natural products, fell out of favor with the rise of HTS and combinatorial chemistry. Companies had also not been investigating synthetic macrocycles, because of the difficulty of synthesizing these compounds. The lack of attention to macrocycles in the pharmaceutical industry has been an important reason for its lack of success in addressing challenging targets.

However, more recently the B/C/P strategy for the synthesis of macrocycle-rich chemical libraries, as well as the development of olefin metathesis as a breakthrough method for the synthesis of organic compounds,

have put macrocycles within the reach of synthetic organic chemists and medicinal chemists, including those in industry.

Moreover, as also described in Chapter 2, Ensemble Therapeutics' proprietary DNA-programmed chemistry (DPC) technology enables this company to synthesize large libraries of macrocyclic compounds, which the company claims represent the largest set of synthetic macrocycles in the pharmaceutical industry. Ensemble has screened its libraries for biological activity and claims to have generated leads against high-value targets, including PPIs. Ensemble's has an internal pipeline of drugs in development, among which are inhibitors of PPIs, as well as collaborative programs with Bristol-Myers Squibb and Pfizer that include the discovery and development of PPI inhibitors.

In addition to the novel enabling technologies for small-molecule PPI modulator development discussed in Chapter 2, Aileron Therapeutics and its academic collaborators has been developing agents to modulate PPIs based on its proprietary stapled peptide technology. This technology is discussed in Chapter 7. Aileron now has an internal pipeline of preclinical stapled-peptide PPI modulators. It also has a collaboration with Roche that is aimed at the discovery, development, and commercialization of stapled-peptide agents. The rationale for stapled peptide technology is to bypass the difficulties in the discovery and development of small-molecule PPI modulators, as opposed to overcoming these difficulties via the novel enabling technologies discussed in Chapter 2.

Pharmaceutical and biotechnology companies are moving back into the PPI modulator field

As discussed earlier in this chapter, companies that had been active in the discovery and development of small-molecule PPI modulators in the 1990s and 2000s had largely retreated from the PPI field (except for continuing development of those few compounds that had gone beyond the discovery stage), because of the poor commercial prospects of the field. However, more recently, some companies have been moving back into the PPI modulator development field, based on the new enabling technologies discussed in the last section.

As discussed in Chapter 2, two companies in particular, Ensemble Therapeutics and Forma Therapeutics (both in Eastern Massachusetts), have built technology platforms based on the new enabling technologies for discovering small-molecule PPI modulators. Ensemble's platform is based on its technology for synthesis and screening of macrocycle libraries. Forma's platform is based on a suite of technologies that were listed in Chapter 2. Another Eastern Massachusetts company, Aileron Therapeutics, has a technology platform based on stapled peptides as also discussed in the last section.

Several large pharmaceutical companies – Bristol-Myers Squibb, Pfizer, and Novartis – have alliances with either Forma or Ensemble for PPI modulator discovery (see Table 3). Novartis also has collaborations with academic groups in the PPI modulator field. Other pharmaceutical and biotechnology companies with active direct small-molecule PPI modulator discovery programs include Ajinomoto (in collaboration with Interprotein Corporation), Ligand, Ascenta (in collaboration with Sanofi), and Cephalon/Teva. Roche has a collaborative program with Aileron to discover and develop stapled-peptide PPI modulators. Together with the companies that are developing clinical-stage direct PPI modulators (Table 7) and those developing clinical-stage allosteric chemokine receptor modulators (Table 4), the commercial PPI modulator field now includes a large and growing section of the pharmaceutical/biotechnology industry. With the new strategic importance of PPIs as targets discussed earlier in this chapter, we expect additional companies to enter the field.

With the emergence of eltrombopag (as well as the allosteric chemokine receptor modulators maraviroc and plerixafor) onto the market, and with other clinical candidates such as navitoclax achieving clinical proof of concept in Phase II trials, there is now the potential for several PPI modulators to become marketed drugs. However, whether the new suite of enabling technologies for PPI modulator discovery and development will enable this area to be commercially successful, or to meet the strategic needs of the industry for expanding the universe of targets for drug discovery and developing drugs that address them, remains an open question. As of early 2012, all of the compounds that have been discovered as a result of applying the new enabling technologies discussed in Chapters 2 and 7 are in the research and preclinical stage. It will be necessary for some of these compounds to enter the clinic and achieve proof of concept and reach the market before these questions can be answered. Nevertheless, the PPI modulator field in 2012 is an exciting area that is gaining increasing interest and investment by leading pharmaceutical and biotechnology companies.

Appendix

Abbreviations

ABDF: 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole

ADAM: A Disintegrin And Metalloproteinase

ADME: absorption, distribution, metabolism, and elimination

AKAP: A-kinase anchoring protein

ALL: acute lymphoblastoid leukemia

ALS: amyotrophic lateral sclerosis

AML: acute myeloid leukemia

Apaf-1: apoptotic protease activating factor-1

APC: adenomatous polyposis coli

ATP: adenosine triphosphate

ATR: ataxia telangiectasia and Rad3-related protein

BAK: = Bcl-2 homologous antagonist killer

Bcl-2: B-cell lymphoma-2

B-CLL: B-cell chronic lymphocytic leukemia

Bcl-XL: B-cell lymphoma-extra large

BMS: Bristol-Myers Squibb

BTB: BR-C, ttk, and bab

CADD: computer-aided drug design

cAMP: cyclic adenosine monophosphate

CasR: calcium-sensing receptor

CBF: core binding factor

CDK: cyclin-dependent kinase

CHK1: checkpoint kinase 1

CLL: chronic lymphocytic leukemia

COPD: chronic obstructive pulmonary disease

CRL: Cullin RING E3 ligase

CS: computational solvent

CTCL: cutaneous T-cell lymphoma

DLBCL: diffuse large B-cell lymphoma

DMSO: dimethyl sulfoxide

DOS: diversity-oriented synthesis

DPC: DNA-programmed chemistry

E6-AP: E6-associated protein

ECM: extracellular matrix

EGFP: enhanced green fluorescent protein

EGFR: epidermal growth factor receptor

ELISA: enzyme-linked immunosorbent assay

EPO: erythropoietin

ESA: erythropoiesis-stimulating agent

FADD: Fas-associated death domain

FASL: Fas ligand

FBDD: fragment-based drug design

FBLD: fragment-based lead discovery

FKBP12: FK-binding protein 12

G-CSF: granulocyte colony-stimulating factor

GFP: green fluorescent protein

GPCR: G-protein coupled receptor

Grb2: growth factor receptor-bound protein 2

GRIP: green fluorescent protein-assisted readout for interacting proteins

GSK: GlaxoSmithKline

GSK3: glycogen synthase kinase 3

HCC: hepatocellular carcinoma

HCS: high-content screening

HDAC: histone deacetylase 3

HDM2: human homolog of mouse double minute 2

HPV: human papillomavirus

HTS: high-throughput screening

ICAM-1: intercellular adhesion molecule 1

IgG: immunoglobulin G

IL-2: interleukin 2

IL-2Rα: IL-2 receptor alpha chain

IND: investigational new drug

ITP: idiopathic thrombocytopenic purpura

Kd: dissociation constant

Ki: inhibition constant

KSR: kinase suppressor of RAS

LFA-1: lymphocyte function-associated antigen 1

LLS: Leukemia & Lymphoma Society

mAb: monoclonal antibody

MAPK: mitogen activated protein kinase

MDM2: mouse double minute 2

MM: multiple myeloma

mTOR: mammalian target of rapamycin

NAE: NEDD8-activating enzyme

NCE: new chemical entity

NCI: National Cancer Institute

NCOR2: nuclear receptor co-repressor 2

NEMO: NF-κB essential modulator

NF-κB: nuclear factor kappa B

NMR: nuclear magnetic resonance

NSCLC: non-small cell lung cancer

PDB: Protein Data Bank

PKA: protein kinase A

PKC: protein kinase C

POZ: pox virus and zinc finger

PPI: protein-protein interaction

PTB1B: protein tyrosine phosphatase-1B

Ptc1: Patched

RA: rheumatoid arthritis

RANTES: Regulated on Activation, Normal T Expressed and Secreted

RNAi: ribonucleic acid interference

SAHB: stabilized alpha-helix of Bcl-2 domain

SAR: structure-activity relationship

SBDD: structure-based drug design

SBIR: Small Business Innovation Research

SCLC: small-cell lung cancer

Shh: Sonic Hedgehog

SMRT: silencing mediator of retinoid and thyroid hormone receptors

snRNA: small nuclear ribonucleic acid

SPR: surface plasmon resonance

STAT: signal transducer and activator of transcription

TACE: tumor necrosis factor alpha converting enzyme

T-ALL: T-cell acute lymphoblastic leukemia

TAP: Therapy Acceleration Program

7TM: seven-transmembrane

TNF: tumor necrosis factor

TPO: thrombopoeitin

TRAIL: tumor necrosis factor α-related apoptosis-inducing ligand

UCSF: University of California at San Francisco

UPS: ubiquitin proteasome system

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

References

Chapter 1 references

- Allison M (2009) Bristol-Myers Squibb swallows last of antibody pioneers. Nature Biotechnology, 27 (9): 781–783.
- Arkin MR, Wells JA (2004) Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. Nature Reviews Drug Discovery, 3 (4): 301–317.

- Bauer RA, Wurst JM, Tan DS (2010) Expanding the Range of 'Druggable' Targets with Natural Product- based Libraries: An Academic Perspective. Current Opinion in Chemical Biology, 14 (3): 308–314.
- Bunnage ME (2011) Getting pharmaceutical R&D back on target. Nature Chemical Biology, 7 (6): 335–339.
- Clamp M, Fry B, Kamal M, et al. (2007) Distinguishing protein-coding and noncoding genes in the human genome. Proceedings of the National Academy of Sciences of the United States of America, 104(49): 19428–19433.
- Cressey D (2011) Pfizer slashes R&D. Nature, 470: 154.
- Edwards AM, Isserlin R, Bader GD, et al. (2011) Too many roads not taken. Nature, 470(7333): 163–165.
- Haberman A (2009) Approaches to Reducing Phase II Attrition. Cambridge MA, USA, Cambridge Healthtech Institute Insight Pharma Reports.
- Hopkins AL, Groom CR (2002) The druggable genome. Nature Reviews Drug Discovery, 1 (9): 727–730.
- International Human Genome Sequencing Consortium (2004) Finishing the euchromatic sequence of the human genome. Nature, 431(7011): 931–945.
- Li JW, Vederas JC (2009) Drug Discovery and Natural Products: End of an Era or an Endless Frontier? Science; 325 (5937): 161–165.
- Lipinski CA, Lombardo F, Dominiv BW, Feeney PJ (2011) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Advanced Drug Delivery Reviews, 46 (1-3): 3–26.
- Lowe DB (2011) Drug Seekers. Cell, 146 (1):16–17.
- Moreira IS, Fernandes PA, Ramos MJ (2007) Hot spots – a review of the protein-protein interface determinant amino-acid residues. Proteins, 68 (4): 803–812.

- Nature Chemical Biology editorial (2010) Retooling chemical probes. Nature Chemical Biology 6 (6): 157.
- Newman DJ, Cragg GM (2007) Natural Products as Sources of New Drugs over the Last 25 Years. Journal of Natural Products, 70 (3): 461–477.
- Reuters Top 6 drugs tipped to be biotech products by 2014. Jun 17, 2009. http://www.reuters.com/article/2009/06/17/us-biotech-medicines-idUSTRE55G4PI20090617 (Accessed September 8, 2011).
- Stockwell BR (2011) The Quest for the Cure: The Science and Stories Behind the Next Generation of Medicines. New York, NY, USA, Columbia University Press.
- Wells JA, McClendon CL (2007) Reaching for high-hanging fruit in drug discovery at protein–protein interfaces. Nature 450 (7172): 1001–1009.

Chapter 2 references

- Almholt DL, Loechel F, Nielsen SJ, et al. (2004). Nuclear export inhibitors and kinase inhibitors identified using a MAPK-activated protein kinase 2 redistribution screen. Assay and Drug Development Technologies, 2(1): 7–20.
- Arkin MR, Randal M, DeLano WL, et al. (2003). Binding of small molecules to an adaptive protein–protein interface. Proceedings of the National Academy of Sciences USA, 100 (4): 1603–1608.
- Cencic R, Hall DR, Robert F, et al. (2011) Reversing chemoresistance by small molecule inhibition of the translation initiation complex eIF4F. Proceedings of the National Academy of Sciences U S A. 108(3): 1046–1051.
- Clackson T, Wells JA (1995) A hot spot of binding energy in a hormone-receptor interface. Science, 267 (5196): 383–386.
- Conn PJ, Christopoulos A, Lindsley CW (2009) Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. Nature Reviews Drug Discovery, 8(1): 41–54.
- Cunningham BC, Wells JA (1989) High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. Science, 244 (4908): 1081–1085.

- Davies K (2008) From PlayStation to Protein Surfaces. Bio-It World, February 18, 2008. http://www.bio-itworld.com/BioIT_Content.aspx?id=69970
- DeLano WL, Ultsch MH, de Vos AM, Wells JA (2000) Convergent Solutions to Binding at a Protein-Protein Interface. Science, 287 (5456):1279–1283.
- Dorr P, Westby M, Dobbs S, et al. (2005) Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. Antimicrobial Agents and Chemotherapy, 49(11): 4721–4732.
- Drahl C (2009) Big Hopes Ride On Big Rings. ACS Meeting News: Constraining molecules in macrocyclic rings could help address challenges in drug discovery. Chemical and Engineering News 87(36), 54–57.
- Driggers EM, Hale SP, Lee J, Terrett NK (2008).The exploration of macrocycles for drug discovery – an underexploited structural class. Nature Reviews Drug Discovery, 7(7):608–624.
- Estabrook M (2010) “Targeting Leukemia and Preventing Its Return”. October 29, 2010 http://innovation.virginia.edu/news/300
- Everts S (2008) Piece By Piece. Chemical & Engineering News, 86 (29): 15–23.
- Forma Therapeutics (2009) Press release. FORMA Therapeutics Debuts with 25 Million in Funding – Will Focus on Difficult Cancer Targets. 1/6/2009. http://www.edbi.com/Uploads%5CAttachment%5C30e6bece_1.pdf
- Galloway WR, Isidro-Llobet A, Spring DR (2010) Diversity-oriented synthesis as a tool for the discovery of novel biologically active small molecules. Nature Communications, 1:80. doi: 10.1038/ncomms1081.
- Gorczynski MJ, Grembecka J, Zhou Y, et al. (2007) Allosteric inhibition of the protein-protein interaction between the leukemia-associated proteins Runx1 and CBFbeta. Chemical Biology, 14(10): 1186–1197.
- Graff JR, Konicek BW, Carter JH, Marcusson EG (2008). Targeting the eukaryotic translation initiation factor 4E for cancer therapy. Cancer Research, 68(3): 631–634.
- Haberman A (2009a) RNAi, embryonic stem cells, and technological prematurity. Biopharmconsortium Blog, July 13, 2009. http://biopharmconsortium.com/blog/2009/07/13/10/

- Haberman A (2009b) Bristol-Myers Squibb acquires monoclonal antibody leader Medarex. Biopharmconsortium Blog, September 28, 2009. http://biopharmconsortium.com/blog/2009/09/28/bristol-myers-squibb-acquires-monoclonal-antibody-leader-medarex/
- Hajduk PJ, Galloway WR, Spring DR (2011). Drug discovery: A question of library design. Nature, 470(7332): 42–43.
- Hansen SK, Cancilla MT, Shiau TP, et al. (2005) Allosteric inhibition of PTP1B activity by selective modification of a non-active site cysteine residue. Biochemistry, 44(21): 7704–7712.
- Harvard University, Office of Technology Development (2011) Available technology: Robotnikinin: small molecule inhibitor of sonic hedgehog (Shh) signaling in human cells. http://www.techtransfer.harvard.edu/technologies/tech.php?case=3216
- Kozakov D, Hall DR, Chuang GY, et al. (2011). Structural conservation of druggable hot spots in protein-protein interfaces. Proceedings of the National Academy of Sciences USA 108 (33): 13528–13533.
- Lee CW, Grubbs RH (2001) Formation of macrocycles via ring-closing olefin metathesis. Journal of Organic Chemistry, 66(21): 7155–7158.
- Moerke NJ, Aktas H, Chen H, et al. (2007). Small-molecule inhibition of the interaction between the translation initiation factors eIF4E and eIF4G. Cell, 128(2): 257–267.
- Moreira IS, Fernandes PA, Ramos MJ (2007) Hot spots – a review of the protein-protein interface determinant amino-acid residues. Proteins, 68 (4): 803–812.
- Morton D, Leach S, Cordier C, et al. (2009) Synthesis of natural-product-like molecules with over eighty distinct scaffolds. Angewandte Chemie International Edition English, 48(1):104–109.
- Nielsen TE, Schreiber SL (2008) Towards the optimal screening collection: a synthesis strategy. Angewandte Chemie International Edition English, 47(1): 48–56.
- Oltersdorf T, Elmore SW, Shoemaker AR, et al. (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 435 (7042): 677–681.

- Pagliaro L, Felding J, Audouze K, et al. (2004) Emerging classes of protein-protein interaction inhibitors and new tools for their development. Current Opinion in Chemical Biology, 8(4): 442–449.
- Raimundo BC, Oslob JD, Braisted AC, et al. (2004) Integrating fragment assembly and biophysical methods in the chemical advancement of small-molecule antagonists of IL-2: an approach for inhibiting protein-protein interactions. Journal of Medicinal Chemistry, 47 (12): 3111–3130.
- Sauvé K, Nachman M, Spence C, et al. (1991) Localization in human interleukin 2 of the binding site to the alpha chain (p55) of the interleukin 2 receptor. Proceedings of the National Academy of Sciences USA, 88 (11): 4636–4640.
- Schreiber, SL (2009) Organic chemistry: Molecular diversity by design. Nature, 457(7226): 153–154.
- Shuker SB, Hajduk PJ, Meadows RP, Fesik SW (1996) Discovering High-Affinity Ligands for Proteins: SAR by NMR. Science 274 (5292): 1531–1534.
- Stanton BZ, Peng LF, Maloof N, et al. (2009). A small molecule that binds Hedgehog and blocks its signaling in human cells. A small molecule that binds Hedgehog and blocks its signaling in human cells. Nature Chemical Biology, 5(3):154–156.
- Thermo Scientific (2011a) BioImage Redistribution Assays. http://www.mscience.com.au/view/ts-bioimage/
- Thermo Scientific (2011b) β-catenin Redistribution Assay. http://www.thermo.fr/eThermo/CMA/PDFs/Product/productPDF_8447.pdf
- Thermo Scientific (2011c) p53-Hdm2 RedistributionAssay. http://www.thermoscientific.jp/cellomics/redistribution/docs/p53-Hdm2.pdf
- Tian SS, Lamb P, King AG, et al. (1998). A small, nonpeptidyl mimic of granulocyte-colony-stimulating factor. Science, 281(5374): 257–259.
- Timmerman L (2011) Genentech Scoops Up Tumor-Starving Drug Program From Forma Therapeutics In Rare Deal. XConomy/Boston, June 27, 2011. http://www.xconomy.com/boston/2011/06/27/genentech-scoops-up-tumor-starving-drug-program-from-forma-therapeutics-in-rare-deal/
- van Noort M, Clevers H (2002).TCF Transcription Factors, Mediators of Wnt-Signaling in Development and Cancer. Developmental Biology 244 (1): 1–8.

- Wells JA, McClendon CL (2007) Reaching for high-hanging fruit in drug discovery at protein–protein interfaces. Nature, 450 (7172): 1001–1009.
- Wilson CG, Arkin MR (2011) Small-molecule inhibitors of IL-2/IL-2R: lessons learned and applied. Current Topics in Microbiology and Immunology, 348: 25–59.
- Yu M, Wang C, Kyle AF, Jakubec P, et al. (2011) Synthesis of macrocyclic natural products by catalyst-controlled stereoselective ring-closing metathesis. Nature 479, 88–93.

Chapter 3 References

- Chustecka Z (2011) New Assay for Circulating Tumor Stem Cells May Be Game Changer. Medscape News Today, April 5, 2011. http://www.medscape.com/viewarticle/740232
- Conn PJ, Christopoulos A, Lindsley CW (2009) Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. Nature Reviews Drug Discovery, 8(1): 41–54.
- Davies, SL, Serradell N, Bolos J, Bayes M (2007) Plerixafor hydrochloride. Drugs of the Future, 32(2): 123.
- Dorr P, Westby M, Dobbs S, et al. (2005) Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. Antimicrobial Agents and Chemotherapy, 49(11): 4721–4732.
- Doyle ML, Tian SS, Miller SG, et al. (2003) Selective binding and oligomerization of the murine granulocyte colony-stimulating factor receptor by a low molecular weight nonpeptidyl ligand. Journal of Biological Chemistry, 278(11): 9426–9434.
- Drahl C (2009) Big Hopes Ride On Big Rings. ACS Meeting News: Constraining molecules in macrocyclic rings could help address challenges in drug discovery. Chemical and Engineering News 87(36), 54–57. http://pubs.acs.org/cen/science/87/8736sci1.html
- Dustin ML, Bivona TG, Philips MR (2004) Membranes as messengers in T cell adhesion signaling. Nature Immunology, 5(4): 363–372.

- De Clercq E, Yamamoto N, Pauwels R, et al. (1992) Potent and selective inhibition of human immunodeficiency virus (HIV)-1 and HIV-2 replication by a class of bicyclams interacting with a viral uncoating event. Proceedings of the National Academy of Sciences USA, 89(12): 5286–5290.
- Erickson-Miller CL, DeLorme E, Tian SS, et al. (2005) Discovery and characterization of a selective, nonpeptidyl thrombopoietin receptor agonist. Experimental Hematology, 33(1): 85–93.
- Fulmer T (2010) Targeting chemokines in breast cancer. SciBX 3(4); doi:10.1038/scibx.2010.105 Published online January 28 2010. http://www.nature.com/scibx/journal/v3/n4/full/scibx.2010.105.html
- Ginestier C, Liu S, Diebel ME, et al. (2010) CXCR1 blockade selectively targets human breast cancer stem cells in vitro and in xenografts. Journal of Clinical Investigation, 120(2): 485–497.
- Graf B, Bushnell T, Miller J (2007) LFA-1-mediated T cell costimulation through increased localization of TCR/class II complexes to the central supramolecular activation cluster and exclusion of CD45 from the immunological synapse. Journal of Immunology, 179(3): 1616–1624.
- Hirschler B (2009) Top 6 drugs tipped to be biotech products by 2014. Reuters, June 17, 2009. http://www.reuters.com/article/2009/06/17/us-biotech-medicines-idUSTRE55G4PI20090617
- Horuk R (2009) Chemokine receptor antagonists: overcoming developmental hurdles. Nature Reviews Drug Discovery, 8(1): 23–33.
- Kalatskaya I, Berchiche YA, Gravel S, et al. (2009) AMD3100 is a CXCR7 ligand with allosteric agonist properties. Molecular Pharmacology, 75(5): 1240–1247.
- Kalota A, Gewirtz AM (2010) A prototype nonpeptidyl, hydrazone class, thrombopoietin receptor agonist, SB-559457, is toxic to primary human myeloid leukemia cells. Blood, 115(1): 89–93.
- Li J, Yang C, Xia Y, et al. (2001) Thrombocytopenia caused by the development of antibodies to thrombopoietin. Blood, 98(12): 3241–3248.
- Ligand Pharmaceuticals (2011). EPO and GCSF: Ligand's Cytokine Receptor Drug Discovery Programs. http://www.ligand.com/epo-gcsf
- Shen W, Barr K, Oslob JD, Zhong M (2008) Modulators of cellular adhesion. United States Patent Number 7,314,938. Issued January 1, 2008.

- Tian SS, Lamb P, King AG, et al. (1998). A small, nonpeptidyl mimic of granulocyte-colony-stimulating factor. Science, 281(5374): 257–259.
- Zhong M, Shen W, Barr KJ, et al. (2010) Discovery of tetrahydroisoquinoline (THIQ) derivatives as potent and orally bioavailable LFA-1/ICAM-1 antagonists. Bioorganic and Medicinal Chemistry Letters, 20(17): 5269–5273.

Chapter 4 references

- Ahmad KF, Melnick A, Lax S, et al. (2003) Mechanism of SMRT corepressor recruitment by the BCL6 BTB domain. Molecular Cell, 12(6): 1551–1564.
- Cerchietti LC, Ghetu AF, Zhu X, et al. (2010) A Small-Molecule Inhibitor of BCL6 Kills DLBCL Cells In Vitro and In Vivo. Cancer Cell, 17(4): 400–411.
- Christian F, Szaszák M, Friedl S, et al. (2010) Small molecule AKAP-protein kinase A (PKA) interaction disruptors that activate PKA interfere with compartmentalized cAMP signaling in cardiac myocytes. Journal of Biological Chemistry, 286(11): 9079–909.
- Clapéron A, Therrien M (2007) KSR and CNK: two scaffolds regulating RAS-mediated RAF activation. Oncogene, 26(22): 3143–3158.
- Compton, LA, Hiebert SW (2010) Anticancer Therapy SMRT-ens Up: Targeting the BCL6-SMRT Interaction in B Cell Lymphoma. Cancer Cell, 17(4): 315–316.
- Fasolini M, Wu X, Flocco M, et al. (2003). Hot spots in Tcf4 for the interaction with beta-catenin. Journal of Biological Chemistry, 278(23): 21092–21098.
- Friedberg JW (2011) Diffuse Large B-Cell Lymphoma. http://www.uptodate.com/contents/patient-information-diffuse-large-b-cell-lymphoma-in-adults
- Ghetu AF, Corcoran CM, Cerchietti L, et al. (2008) Structure of a BCOR corepressor peptide in complex with the BCL6 BTB domain dimer. Molecular Cell, 29(3): 384–391.
- Graham TA, Weaver C, Mao F, et al. (2000) Crystal structure of a beta-catenin/Tcf complex. Cell, 103(6): 885–896.

- Huang SM, Mishina YM, Liu S, et al. (2009) Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature, 461(7264): 614–620.
- Leibniz-Institut für Molekulare Pharmakologie (2011). Medicinal Chemistry. http://fmp-berlin.info/rademann.html
- Lepourcelet M, Chen YN, France DS, et al. (2004) Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex. Cancer Cell, 5(1): 91–102.
- Liljas L (2009) Protein Degradation. http://xray.bmc.uu.se/lars/Practicals/FoldndDeg/Degradation.html
- Lygren B, Taskén K (2008) The potential use of AKAP18delta as a drug target in heart failure patients. Expert Opinion on Biological Therapy, 8(8): 1099–1108.
- Minke KS, Staib P, Puetter A, et al. (2009) Small molecule inhibitors of WNT signaling effectively induce apoptosis in acute myeloid leukemia cells. European Journal of Haematology, 82(3): 165–175.
- Poy F, Lepourcelet M, Shivdasani RA, Eck MJ (2001) Structure of a human Tcf4-beta-catenin complex. Nature Structural Biology, 8(12): 1053–1057.
- Sukhdeo K, Mani M, Zhang Y, et al. (2007) Targeting the beta-catenin/TCF transcriptional complex in the treatment of multiple myeloma. Proceedings of the National Academy of Sciences USA, 104(18): 7516–7521.
- Wei W, Chua MS, Grepper S, So S (2010) Small molecule antagonists of Tcf4/beta-catenin complex inhibit the growth of HCC cells in vitro and in vivo. International Journal of Cancer, 126(10): 2426–2436.

Chapter 5 references

- Aghajan M, Jonai N, Flick K (2010). Chemical genetics screen for enhancers of rapamycin identifies a specific inhibitor of an SCF family E3 ubiquitin ligase. Nature Biotechnology, 28(7): 738–742.
- Appel A (2011) Drugs: More shots on target. Nature, 480(7377): S40–S42.
- Ceccarelli DF, Tang X, Pelletier B, et al. (2011) An allosteric inhibitor of the human Cdc34 ubiquitin-conjugating enzyme. Cell, 145(7):1075–1087.

- Cheok CF, Verma CS, Baselga J, Lane DP (2011) Translating p53 into the clinic. Nature Reviews Clinical Oncology, 8(1): 25–37.
- Cohen P, Tcherpakov M (2010) Will the ubiquitin system furnish as many drug targets as protein kinases? Cell,143(5): 686–693.
- Deshaies RJ (2009) Drug discovery: Fresh target for cancer therapy. Nature, 458(7239): 709–710.
- Kaiser P, Su NY, Yen JL, et al. (2006) The yeast ubiquitin ligase SCFMet30: connecting environmental and intracellular conditions to cell division. Cell Division, 1:16.
- Kitagawa M, Aonuma M, Lee SH, et al. (2008) E2F-1 transcriptional activity is a critical determinant of Mdm2 antagonist-induced apoptosis in human tumor cell lines. Oncogene. 2008 27(40): 5303–5314.
- Kojima K, Burks JK, Arts J, Andreeff M (2010) The novel tryptamine derivative JNJ-26854165 induces wild-type p53- and E2F1-mediated apoptosis in acute myeloid and lymphoid leukemias. Molecular Cancer Therapeutics, 9(9): 2545–2557.
- Kussie PH, Gorina S, Marechal V, et al. (1996) Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science, 274(5289): 948–953.
- Li M, Brooks CL, Wu-Baer F, et al. (2003) Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. Science, 302(5652): 1972–1975.
- Nobel Prize in Chemistry (2004). Aaron Ciechanover, Avram Hershko, Irwin Rose. http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2004/
- Orlicky S, Tang X, Neduva V, et al. (2010) An allosteric inhibitor of substrate recognition by the SCF(Cdc4) ubiquitin ligase. Nature Biotechnology, 28(7): 733–737.
- Proteostasis Therapeutics (2011) Proteostasis Therapeutics Licenses Novel Protein Clearance Targets, Compounds from Harvard University. http://www.proteostasis.com/news_events/documents/Harvard-Proteostasislicensesrelease26April2011.pdf
- Reed D, Shen Y, Shelat AA, et al. (2010). Identification and characterization of the first small molecule inhibitor of MDMX. Journal of Biological Chemistry, 285(14): 10786–10796.

- Secchiero P, Bosco R, Celeghini C, Zauli G (2011) Recent advances in the therapeutic perspectives of Nutlin-3. Current Pharmaceutical Design, 17(6): 569–577.
- Shangary S, Qin D, McEachern D, et al. (2008). Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. Proceedings of the National Academy of Sciences USA, 105(10): 3933–3938.
- Shangary S, Wang S (2009) Small-molecule inhibitors of the MDM2-p53 protein-protein interaction to reactivate p53 function: a novel approach for cancer therapy. Annual Review of Pharmacology and Toxicology, 49: 223–241.
- Soucy TA, Smith PG, Milhollen MA, et al. (2009) An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature, 458(7239): 732–736.
- Vassilev LT, Vu BT, Graves B, et al. (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science, 303(5659): 844–848.
- Welcker M, Clurman BE (2008) FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. Nature Reviews Cancer, 8(2): 83–93.

Chapter 6 References

- Adams JM, Cory S (2007) The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene, 26(9): 1324–1337.
- Boone DN, Qi Y, Li Z, Hann SR (2011) Egr1 mediates p53-independent c-Myc-induced apoptosis via a noncanonical ARF-dependent transcriptional mechanism. Proceedings of the National Academy of Sciences U S A, 108(2): 632–637.
- Chan FK, Baehrecke EH (2012) RIP3 Finds Partners in Crime. Cell, 148(1-2): 17–18.
- ClinicalTrials.gov (2011) Obatoclax. http://clinicaltrials.gov/search/intervention=Obatoclax
- Danial NN, Korsmeyer SJ (2004) Cell death: critical control points. Cell, 116(2): 205–219.
- Dash R, Richards JE, Su ZZ, et al. (2010) Mechanism by which Mcl-1 regulates cancer-specific apoptosis triggered by mda-7/IL-24, an IL-10-related cytokine. Cancer Research, 70(12): 5034–5045.

- Keller TH, Pichota A, Yin Z (2006) A practical view of 'druggability'. Current Opinion in Chemical Biology,10(4): 357–361.
- Nguyen M, Marcellus RC, Roulston A, et al. (2007) Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. Proceedings of the National Academy of Sciences USA, 104(49): 19512–19517.
- Oltersdorf T, Elmore SW, Shoemaker AR, et al. (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature, 435(7042): 677–681.
- Roberts AW, Seymour JF, Brown JR, et al. (2012) Substantial Susceptibility of Chronic Lymphocytic Leukemia to BCL2 Inhibition: Results of a Phase I Study of Navitoclax in Patients With Relapsed or Refractory Disease. Journal of Clinical Oncology, 30(5): 488–496.
- Shore GC, Viallet J. (2005) Modulating the bcl-2 family of apoptosis suppressors for potential therapeutic benefit in cancer. Hematology; American Society of Hematology Education Program, 2005: 226–30.
- Tse C, Shoemaker AR, Adickes J, et al. (2008) ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. Cancer Research, 68(9): 3421–3428.
- Wyllie AH (2010) "Where, O death, is thy sting?" A brief review of apoptosis biology. Molecular Neurobiology, 42(1): 4–9.
- Zaba LC, Fuentes-Duculan J, Eungdamrong NJ, et al. (2010) Identification of TNF-related apoptosis-inducing ligand and other molecules that distinguish inflammatory from resident dendritic cells in patients with psoriasis. Journal of Allergy and Clinical Immunology, 125(6): 1261–1268.
- Zhai D, Jin C, Satterthwait AC, Reed JC. (2006) Comparison of chemical inhibitors of antiapoptotic Bcl-2-family proteins. Cell Death and Differentiation, 13(8): 1419–1421.

Chapter 7 references

- Bernal F, Tyler AF, Korsmeyer SJ, et al. (2007) Reactivation of the p53 tumor suppressor pathway by a stapled p53 peptide. Journal of the American Chemical Society, 129(9): 2456–2457.

- Bernal F, Wade M, Godes M, et al. (2010) A stapled p53 helix overcomes HDMX-mediated suppression of p53. Cancer Cell, 18(5): 411–422.
- Kim YW, Grossmann TN, Verdine GL (2011) Synthesis of all-hydrocarbon stapled α-helical peptides by ring-closing olefin metathesis. Nature Protocols, 6(6): 761–771.
- Moellering RE, Cornejo M, Davis TN, et al. (2009) Direct inhibition of the NOTCH transcription factor complex. Nature, 462(7270): 182–188.
- Schafmeister CE, Po J, Verdine GL (2000) An All-Hydrocarbon Cross-Linking System for Enhancing the Helicity and Metabolic Stability of Peptides. Journal Of The American Chemical Society, 122(24): 5891–5892.
- Stewart ML, Fire E, Keating AE, Walensky LD (2010). The MCL-1 BH3 helix is an exclusive MCL-1 inhibitor and apoptosis sensitizer. Nature Chemical Biology, 6(8): 595–601.
- Walensky LD, Korsmeyer SJ, Verdine, G (2010). Stabilized alpha helical peptides and uses thereof. United States Patent Number 7,723,469. http://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&d=PALL&p=1&u=%2Fnetahtml%2FPTO%2Fsrchnum.htm&r=1&f=G&l=50&s1=7723469.PN.&OS=PN/7723469&RS=PN/7723469
- Walensky LD, Kung AL, Escher I, et al. (2004) Activation of apoptosis in vivo by a hydrocarbon-stapled BH3 helix. Science, 305(5689): 1466–1470.
- Weintraub A (2011) Aileron CEO Hails Expanded Roche Deal as a Validation of Stapled-Peptide Drug Technology. Xconomy Boston. http://www.xconomy.com/boston/2011/11/17/aileron-ceo-hails-expanded-roche-deal-as-a-validation-of-stapled-peptide-drug-technology/?single_page=true
- Zhang H, Zhao Q, Bhattacharya S, et al. (2008) A cell-penetrating helical peptide as a potential HIV-1 inhibitor. Journal of Molecular Biology, 378(3): 565–580.

Chapter 8 references

- Andreeff M, Kojima K, Padmanabhan S, et al. (2010) A Multi-Center, Open-Label, Phase I Study of Single Agent RG7112, A First In Class p53-MDM2 Antagonist, In Patients with Relapsed/Refractory Acute Myeloid and Lymphoid Leukemias (AML/ALL) and Refractory Chronic Lymphocytic Leukemia/Small Cell Lymphocytic Lymphomas (CLL/SCLL). Abstract. 52nd American Society of

Hematology Meeting, Orlando FL, Monday, December 6, 2010. http://ash.confex.com/ash/2010/webprogram/Paper28708.html

- Bunnage ME (2011) Getting pharmaceutical R&D back on target. Nature Chemical Biology, 7 (6): 335–339.
- Haberman A (2009) RNAi, embryonic stem cells, and technological prematurity. Biopharmconsortium Blog, July 13, 2009. http://biopharmconsortium.com/blog/2009/07/13/10/
- Keller TH, Pichota A, Yin Z (2006) A practical view of 'druggability'. Current Opinion in Chemical Biology,10(4): 357–361.
- Lowe DB (2011) Drug Seekers. Cell, 146 (1): 16–17.
- Stockwell BR (2011) The Quest for the Cure: The Science and Stories Behind the Next Generation of Medicines. New York, NY, USA, Columbia University Press.
- Stockwell BR (2012) Does a new treatment for leukemia herald a new era in drug discovery? Soapbox Science (a community guest blog from nature.com). 15 February 2012. http://blogs.nature.com/soapboxscience/2012/02/15/does-a-new-treatment-for-leukemia-herald-a-new-era-in-drug-discovery