Constrained Peptides in Drug Discovery and Development

Douglas R. Cary, Masaki Ohuchi, Patrick C. Reid, and Keiichi Masuya

*PeptiDream Inc.
3–25–23 Tonomachi, Kawasaki-ku, Kawasaki 210–0821, Japan

(Received June 30, 2017; E-mail: k-masuya@peptidream.com)

Abstract: Constrained peptides, namely macrocyclic and stapled peptides, are receiving increasing attention as a promising class of compounds for the inhibition of protein–protein interactions (PPI). The current state of peptide therapeutics is discussed, including their merits and challenges, as well as recent technological developments that have enabled a new era in peptide research and development. The technology behind PeptiDream’s Peptide Discovery Platform System (PDPS) is described, showing how it can be used to rapidly generate libraries of constrained peptides and obtain detailed SAR information. This technology can provide, with a high rate of success, potent peptide ligands that may be developed as drug candidates themselves, utilized in peptide–drug conjugates (PDC), or converted into small molecule drug leads. The outlook for the field of constrained peptides and their use in the clinic is also described.

1. Introduction

Constrained peptides represent a new class of peptide molecules whose supramolecular structure is controlled via intramolecular covalent bonds, generally to confer upon them biochemical and/or physicochemical properties superior to those of ordinary peptides. Both academia and industry are showing increasing interest in constrained peptides, due to their promise as medicines and tools for drug discovery. The major categories of constrained peptides are macrocyclic peptides and stapled peptides, as illustrated in Figure 1. The related field of foldamers, which can be thought of as conformationally constrained peptides, has been recently reviewed and will not be discussed here. This paper will present an overview of ongoing research and development efforts in this field, with particular focus on our approach toward constrained peptide research and development.

1.1 Why Study Constrained Peptides? PPI Drug Targets

Most investigational and approved drugs to date fall into the broad categories of small molecules or macromolecular biologics, with antibodies, proteins and vaccines representing the predominant forms of approved biologic therapies. Until recently, aside from a small number of natural products, there has been much less progress in designing drugs for the intervening space of medium–sized molecules, defined here as molecules with molecular weights ranging from 500 to 6000 Dal-

Figure 1. Three main categories of peptides (linear, stapled, and macrocyclic) and variations.

Figure 2. Representative PPI inhibitors.
onstrated that protein structural data and fragment based drug discovery techniques (FBDD) could be combined to create a drug that interferes with protein–protein interactions (PPI). With a molecular weight of 868 Daltons, this compound stretches the boundaries of what can be called a small molecule, however the resulting drug can be administered orally.

The next most anticipated classes of small molecule PPI inhibitors are antagonists of the MDM2/p53 and XIAP/Caspase–9 interactions, which have also been pursued for decades, and several of which are now in clinical trials.

Within the well-established field of antibodies, substantial effort is being made in the utilization of smaller antibody epitopes and fragments, intrabodies, nanobodies, monobodies, affibodies, etc. as the minimal key binding elements of full antibodies. In addition, most major biopharmaceutical companies are currently active in antibody–drug conjugate (ADC) research and development, particularly in the field of oncology, and various new hybrids of small and large molecules are being actively examined that defy traditional categorization of molecular size.

Much of the motivation for this push into new molecular modalities is due to the untapped pool of potential PPI drug targets, an interest likely to continue given the current intense industry focus on immuno-oncology, for which a wealth of PPI drug targets are anticipated. It is hoped that a new generation of medium-sized molecules could be used to address some challenging drug targets traditionally considered only for antibodies, while retaining many of the desirable properties of smaller molecules.

1.2 The State of Traditional Peptide Drugs

To address the growing need for medium-sized molecules that can interfere with biologically relevant PPI targets, peptides provide an ideal platform; indeed, they are the molecules used by nature in regulating the cellular machinery. They can be readily synthesized under standard conditions that allow for the incorporation of various amino acid building blocks to provide large, well-defined molecules of variable sizes and reasonable physicochemical properties. However, the modern development of peptides as medicines has been hampered due to PK/ADME limitations, as well as difficulties in synthesis and manufacturing.

To date, a number of peptide molecules have been approved for clinical use in a variety of therapeutic areas, including metabolic disease, antibacterial, antifungal, immunology, oncology, and endocrinology, and a representative sample of these is shown in Figure 3. Insulin, approaching 100 years of clinical use, remains the most important historic example, and even now research and development efforts are ongoing into various new ways of administering insulin besides frequent subcutaneous injection. More modestly sized 8- to 14-amino acid peptides such as cyclosporin A, leuprolelin, octreotide, and daptomycin have been developed since the 1980’s, with cyclosporin A notable as being an orally administered drug. Its oral bioavailability is attributed to its stabilizing, yet flexible, macrocyclic structure reinforced with several N-methylated amide bonds. Currently, a wide variety of peptide drugs are being tested in the clinic, with an intense competition emerging over the past several years related to the development of GLP-1 receptor agonists, and exenatide representing the first of several GLP-1 analogues approved for clinical use by the FDA in 2005.

Despite the existence of these examples, the pace of peptide drug discovery and development has not come close to that for either small molecule drugs or antibodies. Each of these drug classes possesses distinct characteristics that affect their general utility as drugs, which are summarized in Table 1. Small molecules have the longest history in drug discovery, since they are relatively easy to synthesize, optimize, and administer. This comes with the trade-off that they frequently possess off-target biological activity that may translate into unexpected biological effects and toxicity during clinical development. Antibodies, on the other hand, are generated to be highly selective toward their desired target from the start, however, are limited to extracellular targets. Antibodies are also more complicated and expensive to manufacture, and can only be administered by injection. In recent years, antibodies

![Figure 3](J. Synth. Org. Chem., Jpn. 1172)
have established a dominant position among the world’s most effective and profitable drugs, however with the establishment of regulatory pathways toward the approval of biosimilars and an increasingly challenging intellectual property environment, the prolonged profitability previously enjoyed by antibody drugs is expected to undergo major changes.

Traditional peptide drug discovery has centered on the generation of analogues to natural peptides or protein fragments, with candidate molecules being prepared by laborious stepwise synthesis for smaller peptides or fermentation/isolation techniques for larger peptides. The peptides derived from these efforts can possess many of the high activity and potency features of antibodies, but also possess many of their issues in terms of manufacturing and administration routes.

1.3 Advances in Peptide Technology

The field of peptide synthesis experienced some major technical advances in the 1980’s and 1990’s that have enabled the stepwise progress seen in peptide drug development to date. The development of solid-phase synthesis techniques using Fmoc-protected amino acids and standardized coupling conditions, combined with the development of automated peptide synthesis instruments, have made the synthesis of long peptides routine (although not without challenges). Peptides on the order of 60 amino acids are routinely prepared on a small scale, while the joining of over 100 amino acids is also possible but more challenging. While the chemistry involved is conceptually simple, real-world challenges in purification and yield become increasingly complicated with peptide length. In addition, cross-linked peptide chains provide more challenging synthetic targets.

In addition to the brute force chemical approach to synthesis, phage display techniques have also come into common use since the 1990’s, by which individual peptides and even libraries of peptides can by expressed on a large scale. However, phage display has its limitations. It is primarily limited to incorporation of the 20 or so proteinogenic amino acids, and it remains difficult to functionalize and control the three-dimensional structures of the proteins thus expressed. Thus, the diversity of peptide libraries that can be obtained through phage display is somewhat limited.

2. DNA-based Synthesis of Constrained Peptides

To address some of the weaknesses of previous peptide synthesis approaches, the research group of Prof. Hiroaki Suga sought to create a new type of system that would allow for the display of constrained polypeptide libraries based on virtually any combination of natural and non–natural amino acids. The initial key discovery around 2003 was the development of Flexizymes, a class of engineered ribozymes that enable the charging of tRNA with almost any natural or non–natural amino acid. This flexibility with respect to substrate stands in contrast to the naturally occurring aminoacyl–tRNA synthetases (ARSs), which are highly specific for their amino acid substrates. This RNA catalyst and substrate system drastically expanded the structural diversity of non–natural acyl–tRNAs, enabling the generation of a wide variety of constrained peptide libraries based on a translation system and corresponding DNA sequences. The company PeptiDream Inc. was founded in 2006 to commercialize this technology and library system for use in drug discovery.

The combination of an acyl–tRNA library with genetic code reprogramming allows for the synthesis of peptides with structures and compositions well beyond those found in nature. As shown in Figure 4, the removal of a given amino acid and its cognate ARS from the canonical codon table allow it to be replaced with a non–natural replacement (Naa1, Naa2, etc.). To date, we have used a wide variety of non–natural amino acids in such systems, such as those containing non–natural sidechains, d–amino acids, N–methylated amino acids, those containing reactive cross–linking groups, and peptoids. This has enabled the synthesis of a wide variety of large, highly diverse peptide libraries. While this technology can be used in the preparation of linear, stapled, and macrocyclic peptide libraries, we focus on macrocyclic peptides in this paper as they have provided some of the most significant results and thus have received the most focus to date.

To utilize this technology, we have developed the Peptide Discovery Platform System (PDPs) for the rapid generation and screening of libraries, followed by the selection of potent hit compounds. While beyond the scope of this review, our TRAP (transcription–translation coupled with association of puromycin linker) display system has been described in detail elsewhere. As illustrated in Figure 5, a random DNA library of greater than 10^24 members is prepared, and translated into a peptide library that is encoded by its corresponding DNA. The DNA–hybridized peptides are screened for binding affinity against their desired target protein, as well as counter–screened against off–target proteins for which binding is not desired. PCR is used to identify the most potent binders, and this process of hit selection is repeated over several rounds. Typically, this results in the identification multiple macrocyclic peptide hits that bind selectively to their target of interest with initial K_d values under 100 nM, often under 10 nM or sub–nM.

<table>
<thead>
<tr>
<th>Mol. Wt.</th>
<th>Small molecules</th>
<th>Traditional peptides</th>
<th>Constrained peptides</th>
<th>Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity</td>
<td>medium-high</td>
<td>high-very high</td>
<td>very high</td>
<td>very high</td>
</tr>
<tr>
<td>Selectivity</td>
<td>low</td>
<td>high</td>
<td>very high</td>
<td>very high</td>
</tr>
<tr>
<td>Intracellular targets</td>
<td>possible</td>
<td>generally not possible</td>
<td>possible</td>
<td>not possible</td>
</tr>
<tr>
<td>PPI inhibition</td>
<td>difficult, but possible</td>
<td>possible</td>
<td>possible</td>
<td>possible</td>
</tr>
<tr>
<td>Plasma stability</td>
<td>low-medium</td>
<td>very low-low</td>
<td>medium-high</td>
<td>very high</td>
</tr>
<tr>
<td>Oral administration</td>
<td>possible</td>
<td>generally not possible</td>
<td>possible</td>
<td>not possible</td>
</tr>
<tr>
<td>Toxicity/side effects</td>
<td>high</td>
<td>low</td>
<td>low</td>
<td>low</td>
</tr>
<tr>
<td>Manufacturing cost</td>
<td>low</td>
<td>low-medium</td>
<td>low-medium</td>
<td>high</td>
</tr>
</tbody>
</table>
3. SAR Studies of Constrained Peptides

Using the above peptide display and selection system, structure–affinity relationship (SAR) data can be rapidly collected for existing libraries, with additional libraries synthesized as needed to further expand the range of derivatives available. Initial hit validation is conducted by binding assay, followed up by confirmation of in vitro activity. With the PDPS technology, validated hits can be obtained and SAR trends uncovered before any traditional stepwise peptide synthesis needs to be conducted. Ultimately, once a promising lead series is identified, traditional solid-phase peptide synthesis techniques are utilized to further progress derivative synthesis of individual compounds on a larger scale.

Figure 6 shows an example of the sort of SAR trends that can be elucidated based on such studies. In this case, each amino acid of a 15–amino acid cyclic peptide was replaced in turn with L–alanine. ELISA assay was used to confirm that certain amino acids and/or non-natural structures were essential to high affinity binding, while others were less important.

In this manner, we can readily replace the original amino acid residues of any peptide with a wide variety of natural and non-natural amino acids. This also allows for the rapid improvement of compound binding affinity using relatively routine peptide synthesis techniques. In a short time, essential and non–essential structural elements can be established just as easily, if not more so, than they would be for small molecules.

4. PeptiDream Approach to Drug Discovery Using PDPS

Our unique approach to drug discovery is outlined in Figure 7. In comparison to traditional high throughput screening (HTS) campaigns for small molecule hits, which can take approximately a year or so to complete, PDPS delivers potent hits within 1–2 months with high target selectivity. These can be quickly optimized by peptide chemistry within a couple more months to yield macrocyclic peptides with selective low–nM to pM binding affinity for their desired target.

One of the major limitations of traditional HTS is that after investing a lot of time and resources into running the...
screen, the quantity and quality of the hits is highly variable and difficult to predict; some campaigns yield no usable hits, while others yield an overwhelming number of weak compounds. Using PDPS, we have generally been able to deliver potent hits for about 90% of the protein targets we have tested to date, even for targets widely deemed “undruggable”. The number and variety of hits can be increased as needed through repeated cycles of compound selection, with each selection cycle providing additional potent ligands. This enables the drug discovery team to quickly address the most crucial issue in early target-based drug discovery, pharmacological target validation at an early stage with a high quality chemical probe, in vitro and potentially in vivo. Structural biology also becomes an invaluable tool from this point, with crystallographic data further clarifying the binding mode and site of the peptide macrocycle. Depending on the target and selection conditions, hit peptides can be identified that bind to different epitopes/sites on the protein surface, providing the potential for target inhibition via different modes of action.

Once sufficient target validation has been conducted, the macrocyclic peptide tool compounds thus obtained can be progressed in essentially two different directions: development of a peptide-based drug or conversion of the hit into a non-peptidic small molecule lead. We discuss these cases, separately, below.

5. Development of Constrained Peptides as Drugs

Depending on the biological target, disease, and lead molecule in question, there are cases in which it may be decided to optimize and develop the macrocyclic compound for clinical use as a peptide-based or peptide-like therapeutic. Among the most challenging aspects of peptide therapeutic development remains the separate, but related, issues of cell permeability and oral bioavailability. Aside from cyclosporin A, as mentioned above, most peptides and constrained peptides to date are not suitable for oral administration or intercellular targets. These subjects are currently the focus of much current research in academia and industry, with ideas just beginning to come together on how to design peptides that can be used for a wider range of drug targets and dosing options. We refer the reader to recent reports on these subjects for an introduction to the ongoing discussion.11 In general, a balance is needed between

---

**Figure 6.** Establishment of SAR by PDPS: amino acid residues critical for protein binding.

**Figure 7.** Comparison of the PeptiDream approach to drug discovery using PDPS vs. the classic small molecule approach.
the high affinity and stability afforded by a constrained structure and enough flexibility and solubility to allow transport between aqueous solution and lipophilic membranes through conformational change (so-called "chameleonic properties"). Oral dosing is generally more feasible for smaller constrained peptides. Traditional approaches have tended to rely on the incorporation of N-methylated amino acids and D-amino acids, as well as cyclization, to reduce overall peptide polarity and increase metabolic stability.

The biopharma industry is currently experiencing a surge in research and development related to antibody-drug conjugates (ADCs) as a means for selectively directing highly potent drugs (mostly anticancer) to target specific cells. At Peptidream, we are extending our technology for constrained peptide discovery to include the discovery and development of peptide-drug conjugates (PDCs). As with antibodies and ADCs, frequently the utility of both the peptide itself and its corresponding drug conjugates are studied in parallel. While the newer field of PDCs is just beginning to take off, there are several reasons to expect the clinical use of PDCs to compare favorably to ADCs (Table 2). Expected advantages for PDCs are well-defined structures and structural diversity of conjugated molecules because they can be readily prepared by total chemical synthesis. Also, the substantially reduced size of PDCs should allow for low dose of administration as well as low immunogenicity. Our initial studies of PDC applications began with the identification of HER2-binding macrocyclic peptides and the preparation of DM1 conjugates of these. This provided us with the opportunity to compare these conjugates to the approved ADC drug Kadcyla (trastuzumab+DM1). PDCs represent a field with the potential for significant progress compared to ADCs, and our work in this area continues.

6. Macrocycles as Starting Points for Small Molecule Discovery

For many drug discovery programs, the ultimate goal is the development of a small molecule oral drug; however, constrained peptides can still play a useful role in this regard. As mentioned above, the macrocyclic hit compounds generated by PDPS can reveal unexpected binding sites and/or protein conformations, including PPI hotspots often not addressed by small molecules. By combining SAR information (Figure 7) and structural biology information (Figure 8), it becomes apparent which portions of the peptide are necessary for protein binding and which are not. For instance, as shown in Figure 8, a large fraction of the macrocycle is exposed to solvent, rather than interacting with the target protein. This provides the medicinal chemist with the necessary information to begin the rational design of new small to medium-sized ligands for their drug target. Such drug discovery could be approached via peptidomimetic modifications to the macrocycle, or by searching for non-peptidic organic molecules that can recapitulate key interactions found between the protein and macroyclic ligand. In the meantime, biologists can utilize the peptide macrocycle in target validation experiments.

7. Constrained Peptide Drug Development

Several companies, mostly biotechnology and small biopharma organizations, are actively pursuing the discovery and development of constrained peptides. Table 3 shows most of the major companies currently involved, and summarizes the different technologies they are using and the larger biopharmaceutical companies with which most of them are partnered. This list does not include the many compound suppliers that currently offer so-called peptide, macrocyclic, or natural product libraries for sale and/or HTS use.

It is worth noting that most of these constrained peptide...
companies began operations around 2005 and after, and the first generation of development projects have just recently begun to enter Phase I and II clinical trials. We anticipate the number of constrained peptides, and middle-sized molecules derived from them, to develop more rapidly over the next few years.

8. Conclusion

The field of constrained peptides is encountering a period of tremendous growth, largely fueled by the need to target protein–protein interactions, which have traditionally been difficult to inhibit with small molecules. In addition, recent technological advances in peptide synthesis technology now allow the rapid synthesis, screening, and optimization of peptides. In this paper, we have provided an overview of the field of constrained peptides and highlighted the PepTid Dream PDPS technology platform, which enables the incorporation of a wide variety of non-natural amino acids into various constrained peptide architectures, of which macrocycles are proving the most immediate value. Libraries of such macrocyclic compounds can be rapidly generated and selected for binding to a specific protein target, substantially shortening the time required to launch drug discovery projects and increase the probability of obtaining druggable hits. Such constrained peptides can be developed as drugs themselves, conjugated to other small molecule drugs in the form of peptide-drug conjugates, or utilized as starting points for the design of non-peptide drug leads. Activity in this field has just begun to take off in the last ten years or so, and with the recent entry of several drugs into clinical trials, we expect this to be the start of a more productive new era in drug discovery and development.

Acknowledgements

The authors would like to express their appreciation to Dr. Takane Yokotagawa for providing pre-publication structural data, the details of which will be published in due course.

References


2) (a) Tsuchikama, K.; An, Z. Drug Discov. Today 2016, 21, 1642. (b) Cromm, P. M.; Spiegel, J.; Grossmann, T. N. (c) Cardote, T. A. F.; Ciulli, A. 


**PROFILE**

**Douglas R. Cary** obtained his B.S. in 1991 (Univ. Illinois Urbana–Champaign, Prof. William Pirkle) and Ph.D. in 1995 (Univ. California Berkeley, Prof. John Arnold), followed by a postdoctoral fellowship through 1997 at the University of Oxford (Prof. Dermot O’Hare). At Lawrence Livermore National Laboratories, he participated on the development of a continuous glucose sensor for diabetes (with MiniMed/Medtronic) through 2001. He conducted chemistry in support of clinical development at Pharmacia until 2002. At Sunesis Pharmaceuticals, he led FBDD efforts on PPI drug discovery projects with Biogen and Merck until 2005. As Principal Scientist and Associate Director at Takeda Pharmaceutical, he led medicinal chemistry efforts in oncology drug discovery through 2016. He joined PeptiDream Inc. in 2016 as Director of Chemistry, leading medicinal chemistry efforts to progress constrained peptides into drugs.

**Masaki Ohuchi** obtained his M.S. in 2005 (Japan Advanced Institute of Science and Technology, Prof. Takahiro Hohsaka) and his Ph.D. in 2009 (Univ. Tokyo, Prof. Hiroaki Suga). In the Suga laboratory, he participated in the design and screening of hyperglycosylated glycoproteins in collaboration with Prof. Yasuhiro Kajihara. He joined PeptiDream Inc. in 2013 as Director of Discovery, leading various projects, including PDCs.

**Patrick C. Reid** obtained his B.S. in 1998 (Univ. Vermont), and his Ph.D. in 2003 (Prof. Ta–Yuan Chang) along with Graduate Business Training at Dartmouth College. He is currently the Senior Vice President and Head of R&D, Managing Director, and a member of the Board of Directors at PeptiDream Inc., a publicly traded Tokyo-based biopharmaceutical company. At PeptiDream, Dr. Reid directs all R&D programs, both internal and external, and also directs all international business development aspects of the company. Prior to cofounding PeptiDream in 2006, he was an Associate Professor in the Department of Chemistry and Biotechnology, and also in the Department of Molecular Biology and Medicine, at the University of Tokyo and has published research spanning a variety of research fields, including atherosclerosis, inflammation, metabolism, neurological disease, and cancer research.

**Keichi Masuya** obtained his B.S. in 1993 (Tokyo Science Univ, Prof. Masaaki Ueki) and Ph.D. in 1998 (Tokyo Institute of Technology, Prof. Isao Kawaijima), and Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists (1995–1998). From 1998–2001, he worked at Mitsubishi Pharma in immunology and oncology drug discovery research. He joined Novartis Pharma Japan in 2001, received an Oncology Principal Award in 2004, then moved to Basel Headquarters of Novartis Pharma in 2005. He led various kinase and PPI projects as a project leader. He received a VIVA award as a Novartis leading scientist in 2012. He managed several development compounds in the field of oncology. He joined PeptiDream Inc. in 2014 as Head of Chemistry, as was promoted as Vice President and Board Member in 2015.