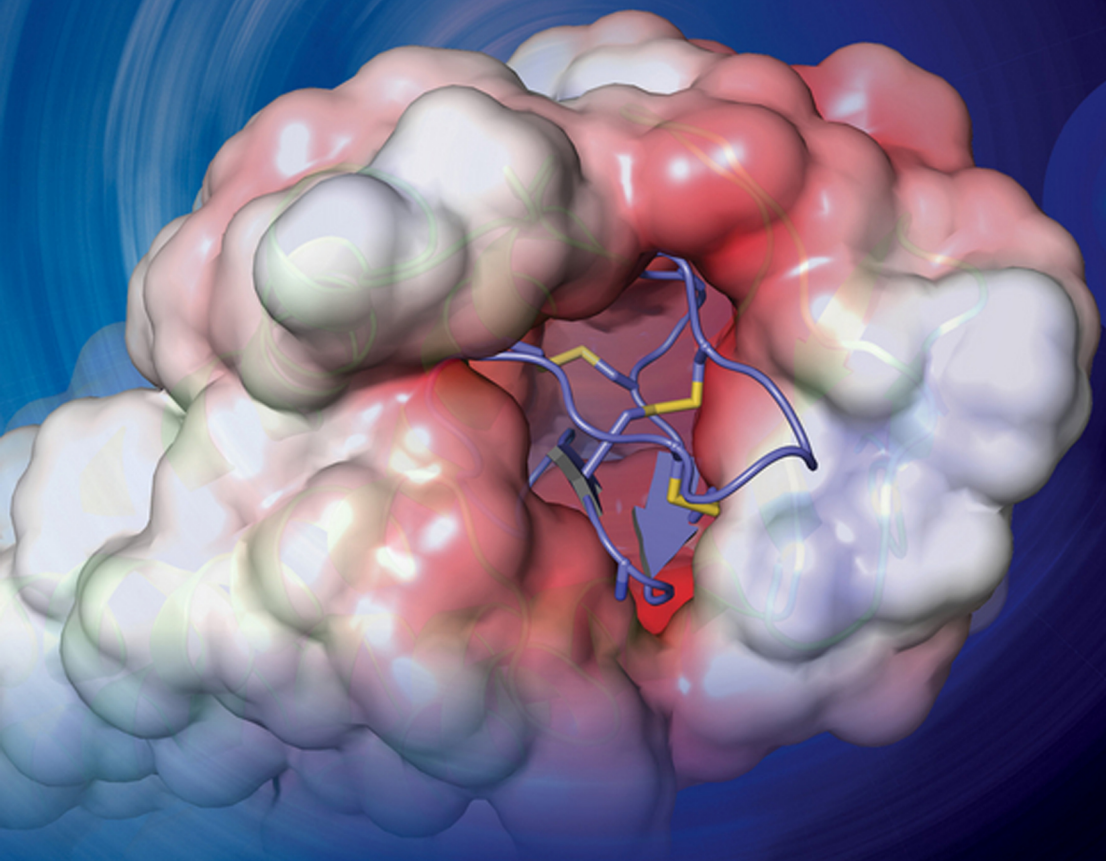
Peptide Chemistry and Drug Design



Edited by

Ben M. Dunn

WILEY

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PREFACE

This book is result of many conversations with peptide scientists at a variety of meetings, including American Peptide Society Symposia, meetings of the European Peptide Society, the Japanese Peptide Society, and the Australian Peptide Society. Some of these conversations were with the authors of the chapters in this book. One additional influence was a meeting in Dubai, where I had an excellent dinner with Waleed Danho, then with Roche Nutley. Waleed had given an excellent talk about the value of peptide chemistry and peptides as elements in the drug-discovery process. Over a delicious dinner of baked fish and many other courses, we discussed the history of drug discovery and the role that peptides have played in the past. Waleed made the strong point that peptides still have great value in the discovery process and, with appropriate methods to deal with delivery and metabolism issues, can provide excellent drugs for the future.

At around this time, I was contacted by Jonathan Rose of John Wiley & Sons who asked if I would be interested in editing a book on peptides and drug discovery. Sometimes life provides a nice juxtaposition of ideas and I immediately accepted the invitation. Over the following years, I spoke with many scientists, emailed some more, and worked on putting together the chapters for this book. I want to thank Jonathan as well as Kari Capone of John Wiley for their patience and advice over the years it took to bring this together.

The book starts with a chapter provided by Nader Fatouhi, discussing the current state of peptides in drug discovery. I heard Nader speak at the 23rd American Peptide Symposium in the Kona region of the Big Island of Hawaii. As I felt that his presentation provided an update on the thoughts first revealed to me by Waleed Danho, I asked Nader to contribute the opening chapter of the book, as this sets the stage for what follows. In his chapter, Nader discusses the rising importance of peptides as

molecules for drug development as well as the issues facing scientists in this field, including cell penetration, stability, and targeting. Tools and techniques are available to address each of these limitations at this time.

Chapter 2 was contributed by Fernando Albericio and colleagues. This presents modern methods of peptide synthesis in a very readable format. Included are sections on solid supports for solid-phase peptide synthesis, which dominates most research level approaches, linkers, protecting groups, methods for peptide-bond formation, and a variety of methods to modify peptides to limit metabolism. In all cases the latest reagents and techniques are featured, thus making this chapter a great starting point for scientists starting out in the peptide field. The authors go on to discuss synthesis of peptides in solution, which still has great value in certain applications, including production of peptides in bulk. In addition, the combination of both solution- and solid-phase methods is discussed for cases where fragment condensation is used to prepare ever larger peptides. This discussion includes native chemical ligation, which permits selectively linking N-termini and C-termini of fragments, and which has several variations with more coming each year. The chapter concludes with a very valuable discussion of separation methods and methods for the analysis of the products of peptide synthesis. Again, this chapter is recommended as a great starting place for new scientists.

Anamika Singh and Carrie Haskell-Luevano have provided Chapter 3 that discusses the important topic of membrane receptors as targets for drug discovery. Due to the vital role of membrane receptors in cell signaling and control of metabolic events, a significant percentage of drugs in current use exert their function by interfering or stimulating binding and signaling events at membrane receptors, also known as G-protein coupled receptors (GPRCs). This chapter provides a catalog of systems where peptides are known to be involved and where it has been shown that synthetic peptides can modulate function. The Haskell-Luevano lab has provided outstanding research on the melanocortin receptors, but this chapter takes a broader approach and discusses a wide variety of these systems, including structural information as known and as modeled by other labs. Anyone involved in aspects of membrane signaling will find this chapter a highly valuable resource for methods, approaches, and strategies for attacking this important area of biology.

Gregg Fields and colleagues present Chapter 4 to introduce the use of peptides as inhibitors of enzymes. In the first part, the authors introduce enzymes and their classification and present several classical examples of the use of peptides to come up with compounds that provide the desired change in enzyme function to overcome a metabolic defect. In a second section, the area of HIV-1 infection and progression to AIDS is described, with emphasis on the value of peptides as modulators of growth and infection. As the human immunodeficiency virus goes through a complicated life cycle, the authors point out that there are multiple targets for approaching therapy and a combination strategy, known as HAART (highly active antiretroviral therapy) has provided the optimal approach to treatment of affected individuals. The Fields lab has made major contributions to discoveries in the area of matrix metalloproteinases and this chapter presents a thorough discussion of this system. The enzymes in this family provide a great example of the development of inhibitors through a process of

discovery of aspects of structure and function that can guide the process. The chapter continues with nice discussions of several other systems where peptide chemistry has been key in new discoveries that have driven the drug-development process.

Jeffrey-Tri Nguyen and Yoshiaki Kiso have provided Chapter 5, which continues the discussion of enzyme inhibitors from the aspect of peptides. The highly productive Kiso lab has led the way in creating a very large catalog of peptide derivatives for use in drug discovery in several systems. They begin this chapter by discussing the advantages and disadvantages of peptides as potential drugs and come down on the side of the beneficial role that peptides play. In particular, they make the important point that the use of peptides can frequently define the pharmacophore, or structural model, which can then be transformed into a small molecule of non-peptide nature for further development as a potential drug. This chapter further focuses on the process of the design of potential inhibitors and reviews the history of discovery from natural sources as well as through *ab initio* design. They discuss the advantages of learning from the natural substrates of an enzyme and introduce the important concept of the transition state analog; the critical role that structural information on the target protein can provide. This chapter provides an excellent discussion of systems where targeting with peptide molecules may provide opportunities for further drug discovery.

Sónia T. Henriques and David J. Craik describe many peptide inhibitors from natural sources in Chapter 6. The introduction to their chapter discusses the value of finding compounds from nature and describes a number of sources, including the antimicrobial peptides from many bacteria. In both bacterial and plant worlds, there is a continual war between competing systems, and this has led to the development through evolution of many natural peptides that serve as defensive molecules. The authors discuss the cyclotides, peptides that are connected end to end and that have multiple disulfide bonds. This arrangement is very stable and the molecules are found in venoms of several species as well as in plants. After this introduction, the authors turn to a discussion of the drug discovery process from their perspective. The chapter continues with an in depth discussion of a variety of systems where many methods are used to modify molecules isolated from nature and where the activity against many targets is tested. The wide diversity of structures and targets is featured in this chapter and the many discoveries have pushed research and drug discovery forward significantly.

Isuru R. Kumarasinghe and Victor J. Hruby have taken on the task of describing methods to limit the metabolism of peptide molecules in humans. This leads to a very detailed discussion of the chemistry of peptide modification. As Victor Hruby is the world leader in this aspect of peptides, the chapter is thoroughly exciting and interesting. A main concern is the digestion of peptides by proteolytic enzymes present in both the digestive tract and the circulation. The first step is to define the pharmacophore residues of a naturally occurring and effective peptide. This will show the absolutely critical functional groups and their stereochemical relationships that must be maintained. Then replacement of some nonessential amino acids by non-natural amino acids, with the D-amino acid isomer, or with peptide-bond isosteres may be sufficient to block degradation by proteases. In addition, cyclization can sometimes provide more stability and also enhance passage of peptides through

the blood–brain-barrier. Other strategies include replacement of specific the amino acids with the *N*-methyl derivatives, with topographically constrained derivatives, or with the halogenated derivatives of aromatic amino acids. Finally, the use of the "multiple-antigenic-peptide" approach where many molecules are attached to a carrier with multiple attachment points can produce molecules that, due to their size, are not recognized by proteases. This chapter emphasizes the role of creative synthetic chemistry is the modification of peptides to achieve stability and bioavailability.

The book concludes with Chapter 8, provided by Jeffrey-Tri Nguyen Yoshiaki Kiso, that discusses the important area of peptide delivery. While progress in the past 50 years has permitted peptide chemists to make almost any sequence of amino acids that is desired in high yield and purity, getting those molecules into humans and into the specific area in the body where they can exert a therapeutic effect is a problem that has not progressed as rapidly. Thus, this chapter is very important for future advances in drug discovery based on peptides. Many of the readers may already be familiar with the Lipinski's Rule of Five that includes recommendations for the size of a molecule, the number of hydrogen bonding atoms, and the lipophilicity. These rules are discussed in this chapter, but much more information is provided regarding solubility, membrane transport, and metabolic stability.

In conclusion, this book provides a primer for anyone in the field of drug discovery and specifically in the area of the use of peptides as molecules for both the discovery phase and, in favorable cases, the final phase of the creation of new molecular entities that can be moved into further studies to evaluate their potential as therapeutic drugs. I want to thank the authors of the chapters for their friendship, for many discussions, and for their excellent writing for this book.

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September 3, 2014

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PEPTIDE THERAPEUTICS

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1.1 HISTORY OF PEPTIDES AS DRUGS

The advent of molecular biology and our understanding of the physiological and pathological functions of peptides, coupled with advances in synthetic methodologies and peptidomimetics, marked the beginning of a new era in peptide and protein therapeutics, with the vision that there should be no limit to what can be produced as therapeutics. During that period a number of great peptide drugs such as Sandostatin, Lupron, Copaxone, and Zoladex were developed with great therapeutic benefit. The number of approved peptide drugs, however, remains low.

It was not until the last decade that we have seen a significant surge in the number of peptide therapeutics on the market (Figure 1.1). While 10 peptides were approved between 2001 and 2010, the current decade has thus far witnessed the approval of six new peptide therapeutics – a remarkable yearly increase [1, 2]. The number of peptides in development is also steadily growing roughly doubling every decade (Figures 1.2 and 1.3), and there are 400–600 peptides in preclinical studies. This is due to the advances made in our understanding of peptide stability, peptide synthesis, and formulation over the last three decades. Although the market share of peptide drugs is still relatively small (about 2% of the global market for all drugs), the approval rate for peptide drugs is twice as fast as the rate for small molecules, and the market is growing similarly at a rate that is twice the global drug market [3, 4].

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Trade name	Generic name	Target	Indication	Year
Forteo	Teriparatide	PTH1R agonist	Osteoarthritis	2002
Fuzeon	Enfuvirtide	Protein–protein inh.	HIV	2003
Prialt	Aiconotide	Ca^{2+} channel inh.	Pain	2004
Byetta	Exenatide	GLP-1 R agonist	T2 diabetes	2005
Symlin	Pramlintide	Calcitonin agonist	T1/T2 diabetes	2005
Somatuline	Lanreotide	SST agonist	Acromegaly	2007
Nplate	Romiplostim	Thrombopoietin agonist	Haematology	2008
Egrifta	Tesamorelin	GHRF agonist	Lipodystrophy	2010
Victoza	Liraglutide	GLP-1 R agonist	T2D	2010
Bydureon	Exenatide LAR	GLP-1 R agonist	T2 diabetes	2011
Surfaxin	Lucinactant		IRDS	2012
Omontys	Peginesatide	Erithropoeitin analog.	Anemia	2012
Signifor	Pasireotide	Somatostatin analog	Cushing's disease	2012
Kyprolis	Carfilzomib	Proteasome inhibitor	Multiple myeloma	2012
Linzess	Linaclotide	Guanidyl cyclase 2C agonist	IBS-C and CIC	2012
Gattex	Teduglutide	Gluc-like peptide analog	SBS	2012

Figure 1.1 Peptide therapeutics marketed since 2002. (*See insert for color representation of this figure*.)

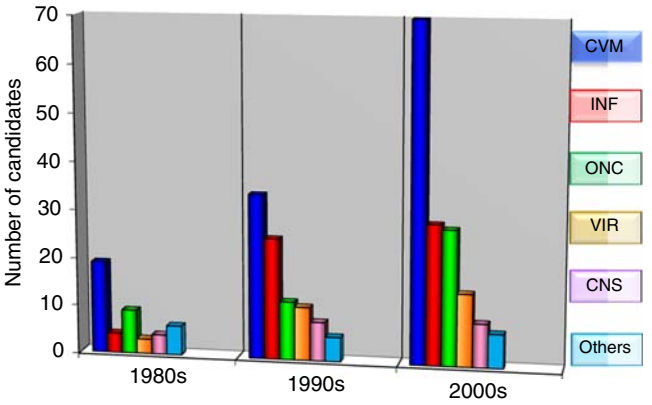

Figure 1.2 Peptides in development over the last three decades. (*See insert for color representation of this figure*.)

While encouraging, the potential for peptide therapeutics is far greater than what it is today.

1.2 FACTORS LIMITING THE USE OF PEPTIDES IN THE CLINIC

A number of factors have thus far limited the explosion that needs to happen in the peptide field. With the exception of a few peptides, the approved drugs so far target the extracellular compartment, and thus have to compete with biologics. Of the

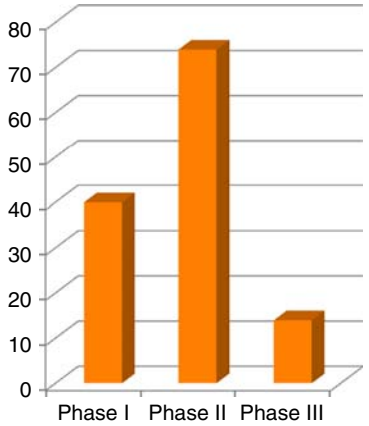

Figure 1.3 Peptides in clinical trials in 2013.

extracellular targets, GPCRs represent the major class, and in most cases, the peptides are agonist. GLP-1 represents one-third of these GPCR targets. We have seen a great advance in extending the circulating half-life of the peptides through the use of unnatural amino acids and formulation technologies, but have not yet reached the half-life achieved by antibodies. The delivery of peptides is still in the great majority of cases limited to *i.v.* (intravenous), *s.c.* (subcutaneous), or intranasal. Finally, safety is still a concern as better tissue selectivity is required.

To dramatically heighten their impact, peptides need to access the intracellular space to target protein–protein interactions. These interactions represent a vast source of potential targets with significant biological impact (there are estimated 300,000 such interactions in the cell), and will not in the majority of cases be modulated by small molecules. Peptides and biologics, given their relative size and ability to bind to extended surface areas, are the perfect candidates to inhibit protein–protein interactions. The duration of action of peptides needs to be extended, and while peptides are inherently selective against their targets, they need to more selectively distribute to the desired tissue. Finally, the route of administration needs to be expanded to include oral delivery.

1.3 ADVANCES THAT HAVE STIMULATED THE USE OF PEPTIDES AS DRUGS

The many great technological advances that started over a decade ago in drug delivery, peptide design, and synthesis are now maturing, and will undoubtedly address these key challenges and revolutionize the field over the next decades. Many of the technological advances are already proving that it is possible to make peptides permeable to cells, target tissues, have longer half-lives, and be orally bioavailable.

The discovery that certain peptides can penetrate cells and can, therefore, be an effective therapeutic on their own or alternatively bring other drugs into cells allowed for the first time to imagine targeting the intracellular compartment (Figures 1.4

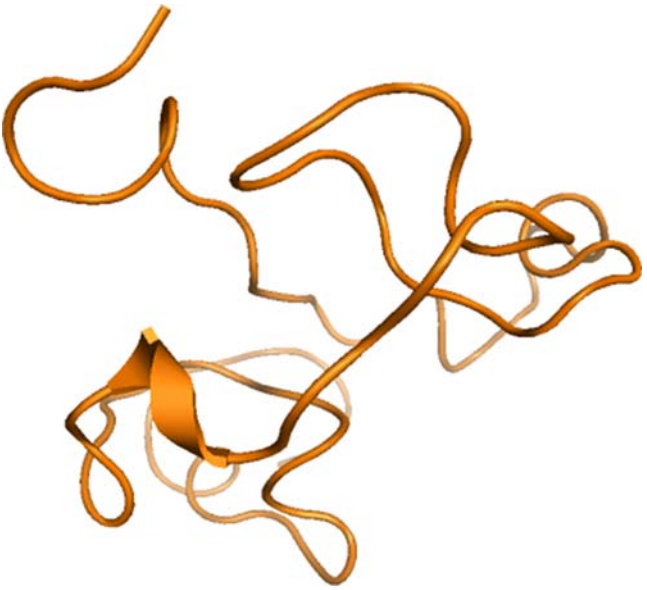

Figure 1.4 HIV Tat.

and 1.5) [5]. HIV-enveloped protein tat was one of the first to be recognized for its cell-penetrating ability and, therefore, its potential use to carry bioactive cargo into the cell [6]. Since 2004, more than 200 peptides carried into cells by tat or other naturally occurring cell-penetrating peptides (CPPs) have been in various phases of development [7]. However, the more recent advances in the understanding of how these peptides cross the cell membrane through endocytosis and/or macropinocytosis [8] has allowed the generation of CPPs with intrinsic biological activity [9–12]. It is now possible to take a CPP sequence and synthetically modify it to introduce the key amino acids of an effector peptide into its sequence and create potent peptide antagonists of an intracellular protein–protein interaction with good pharmacokinetic properties [13].

1.4 DEVELOPMENT OF PEPTIDE LIBRARIES

By looking at the list of CPPs in development, one realizes that they are single cases and have to be synthetically prepared and modified to impart some of the desired stability to be a useful therapeutic. It is hard to compete with the screening of the millions of small molecule compounds in various pharmaceutical companies and more recently in many academic centers.

Until now, the available technologies to screen large libraries of peptides of significant length (possessing secondary structure) would only allow us to generate large libraries of natural amino acid sequences through phage display, and if unnatural

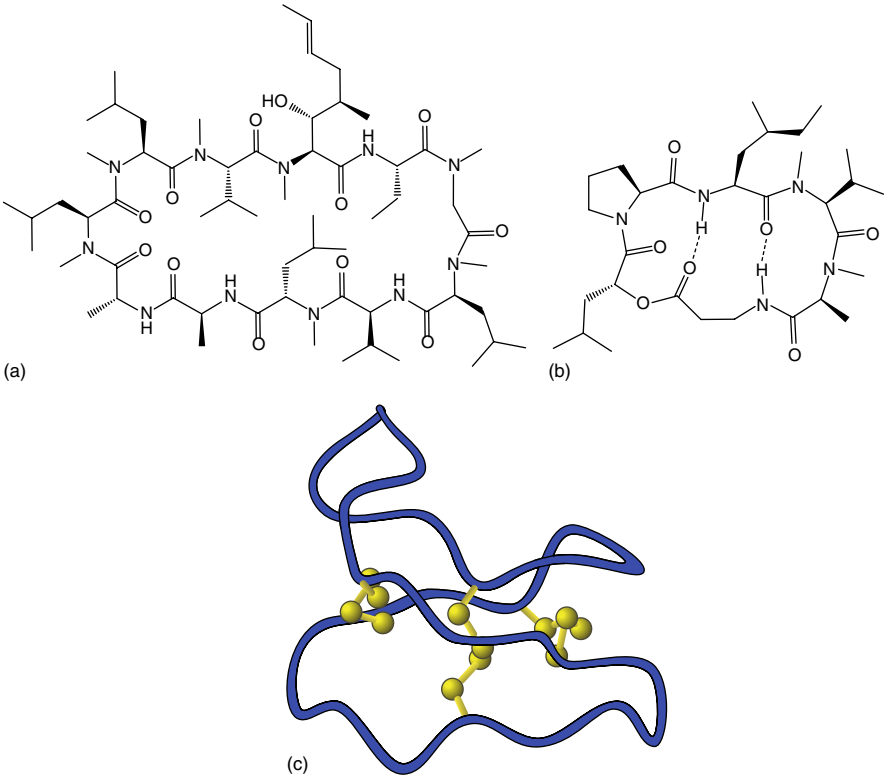

Figure 1.5 Orally stable and bioavailable peptides (a) Cyclosporin. (b) Destruxin. (c) Kalata B.

amino acids were to be introduced, it had to be done with conventional synthetic methodology, and thus be limited to very low numbers of peptides that can be prepared and screened.

Indeed, over the last decade, there has been an explosion of very elegant technologies that now allow the generation of large to extremely large libraries of linear and macrocyclic peptides with unnatural amino acids and unnatural linkers. For the first time, it is possible to engineer stability, cell permeability, and possibly oral bioavailability at once and screen for the desired properties very rapidly. These major advancements have resulted in the generation of a number of companies that are pushing the limits of these technologies to rapidly screen and identify novel peptide therapeutics against protein–protein interaction targets (Figure 1.5).

Ensemble therapeutics utilizing their DNA-programmed chemistry can generate million-member libraries of small macrocycles with MW of 500–1500. On screening these libraries, they have identified potent and orally bioavailable small molecule inhibitors of IL17 [14]. Through medicinal chemistry optimization, they have now identified picomolar inhibitors with good properties [15]. PeptiDream utilizing Professor Suga's mRNA display technology [16] are generating up to trillion-member

libraries of larger macrocycles mimicking cyclosporin. These peptides contain a combination of natural, unnatural, and *N*-methyl amino acids and exhibit good physicochemical properties and membrane permeability [17]. Ra Pharmaceuticals also uses a mRNA display technology developed by Jack Shoztac to generate very large libraries of macrocycles containing unnatural amino acids. They recently presented on their discovery of potent antagonists of mcl-1 and Ras with good cell permeability [18].

1.5 MODIFICATION OF PEPTIDES TO PROMOTE STABILITY AND CELL ENTRY

The recent focus on another class of macrocycles, containing multiple disulfides, has generated a lot of excitement in maintaining the stability and membrane permeability of the cyclotide kalata B1, or the knottins (the uncyclized version of cyclotides), in order to create potent peptide drugs. David Craik and colleagues at Cyclotide are systematically exchanging the various loops present on cyclotides with sequences that have important biological function [19]. Recently, the introduction of a myelin oligodendrocyte glycoprotein sequence into a cyclotide resulted in a potent peptide in preventing disease progression in a mouse model of MS [20]. Protagonist is taking advantage of the oral stability of the disulfide-rich peptides for local gut delivery of IL6R antagonists for the treatment of irritable bowel disease (IBD). Moreover, novel technologies developed for the rapid generation and screening of extremely large libraries of knottins and cyclotides will undoubtedly have a major impact on this class of peptide therapeutics. Of note is the Intein-based technology from Julio Camarero capable of introducing unnatural amino acids to facilitate screening [21]. Sutro and MitiBio also have very sophisticated and efficient biosynthetic methods to generate very large libraries.

Finally, Verdine and Wollensky and colleagues [22, 23] as well as the investigators at Aileron Therapeutics have developed a novel stapling technology that imparts stability and membrane permeability to alpha helical structure. Using this technology, Aileron Therapeutics were able to discover very potent dual MDM2/MDMx antagonists with low nanomolar activity in cells and excellent pharmacokinetic properties, resulting in excellent antitumor activity in a mouse xenograft model [24]. Even more interesting is the extended efficacy ATSP-7041 exhibits in cells. While the small molecule MDM2 antagonist showed activity over 24 h, ATSP-7041 was still active beyond 48 hours in the same experiment. This is due to the fact that once the peptide enters the cell, the major elimination pathway is through enzymatic catabolism. Not only can stability be tuned for circulating half-life, it can also be tuned to withstand cellular catabolism to lengthen the desired efficacy. This could offer a significant advantage over (small) molecules that passively diffuse through the cell membrane. Additionally, using the same technology, a GHRH antagonist with much extended half-life was discovered and is currently in Phase I clinical trial [25].

1.6 TARGETING PEPTIDES TO SPECIFIC CELLS

One of the greatest challenges in drug discovery is the safety of therapeutics. Main reasons for diminished safety are selectivity against the target and tissue/cell specificity. If one could direct a therapeutic to only the site of pathology, then the therapeutic window of the agent increases and correspondingly decreases the side effects. Peptides, due to their specificity against receptors, are perfect candidates to be able to home into one type of cell/tissue versus another. There has been a tremendous amount of progress in identifying homing peptides (cell-penetrating as well as nonpenetrating) that can then be conjugated to a cargo to deliver it to a specific organ [26].

In vivo phage display by Pasqualini and colleagues marked the discovery of the first homing peptide that was able to selectively target the blood vessel of brain and kidney [27]. Since then a number of peptides have been identified that target many other tissues [28]. Arap and colleagues were then the first to perform phage display in humans and discovered a homing peptide to IL11Ra that expresses over 100-fold more on prostate cancer cells versus normal cells [29, 30]. Arrowhead Research is currently in Phase I proof of targeting with a peptide drug conjugate utilizing this homing peptide. Recently, Wen et al., at the Dana Farber, published their first Phase I study result on GRN1005, a peptide drug conjugate that targets the low-density lipoprotein-related protein-1, which mediates blood brain barrier transcytosis. GRN1005 successfully crosses the BBB and delivers its cargo [31].

1.7 FORMULATIONS TO IMPROVE PROPERTIES

While the above advances have and will have significant impact, the ability to administer peptides by the oral route will truly allow them to compete with small molecules and biologics as first line therapies. The majority of advances in this area have been the result of very interesting formulation strategies. A number of companies, including ArisGen, Axcess, Chiasma, Emisphere Tech., Enteris Pharmaceuticals, Lipocine, and Merlion Pharmaceuticals, have had successes in enhancing the oral bioavailability of some peptide therapeutics. They employ a combination of stabilizers, absorption enhancers, and carriers to achieve this. The main mode of absorption is through the paracellular space. However, the bioavailability of the peptides formulated remains relatively low.

While significant, cyclosporin remains the only marketed peptide drug that is administered orally and absorbed into the systemic environment. Learning from nature and systematic studies on macrocyclic peptides will have a tremendous impact in discovering peptide drugs with inherent oral bioavailability that could then be enhanced through formulation to achieve bioavailabilities, which would compete with small molecules. As mentioned earlier, PeptiDream and Ra Pharmaceuticals are generating large libraries of macrocyclic peptides mimicking the core structure of cyclosporin. Ensemble therapeutics are generating small macrocylic structures with molecular weights between 500 and 1500 and have already identified an orally

bioavailable IL17 R antagonist. Professors Horst Kessler and Locky are doing the first systematic studies on small cyclic peptides to understand the effect of hydrogen bonding and structure on bioavailability [32, 33]. Their work will undoubtedly form the basis of rational designs of orally active peptide drugs.

In conclusion, the great technological advances over the last two decades are well poised to have a major impact on revolutionizing the field of peptide therapeutics. For the first time, tools are available to create stable, cell permeable, long lasting, and orally bioavailable peptides, allowing them to compete with small molecule drugs and biologics, and thus become first line therapies for many diseases with unmet medical needs.

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2

METHODS FOR THE PEPTIDE SYNTHESIS AND ANALYSIS

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2.1 INTRODUCTION

Peptides as drugs show unique characteristics (high biological activity, high specificity, and low toxicity) thereby making them particularly attractive therapeutic agents [1]. However, the role of peptides in drug discovery has suffered *ups* and

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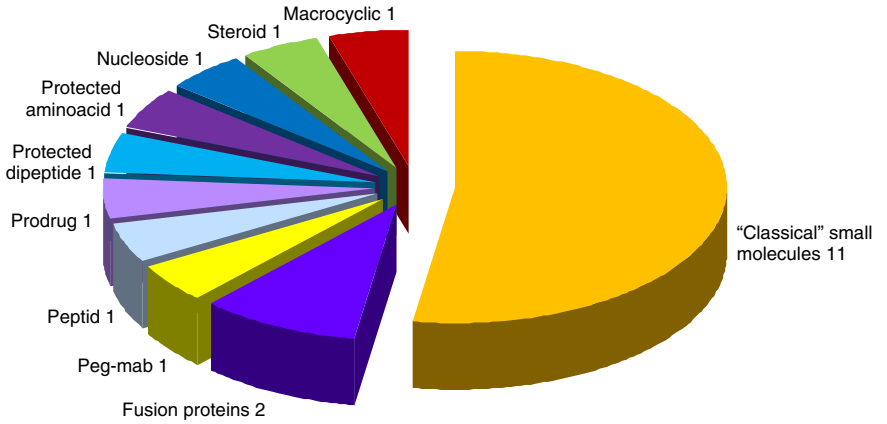

Figure 2.1 Distribution by chemical structure of the new drugs approved by the FDA in 2008. (*See insert for color representation of this figure*.)

downs during the last four decades. A first analysis of the new chemical entities (NCEs) accepted by the Food and Drug Administration (FDA) indicated that while 53 NCEs were introduced as drugs in 1996, only 17 were introduced in 2002. This number increased to 31 in 2004, but decreased again in 2005 with just 18 new drugs, 17 in 2007, and a slight increase to 21 in 2008 (Figure 2.1) [2, 3]. An analysis of these 21 drugs approved in 2008 indicated that almost 50% of the new drugs can be considered *nonclassical*, in the sense that they are *nonclassical small molecules.*

Interestingly, peptides represent approximately 20% of the total number of drugs approved by the FDA in 2008 [3]. Thus, Romiplostim from Amgen, which is a thrombopoietin receptor agonist, is a fusion protein conjugated with a 41 amino acid peptide, containing two disulfide bridges. Degarelix from Ferring, which is a gonadotropin-releasing hormone receptor antagonist, is a 10 amino acid peptide. Alvimopan from Adolor, which is a peripherally acting μ-opioid receptor antagonist, is an N-terminal blocked dipeptide. Lacosamide from Schwarz, which selectively enhances slow inactivation of voltage-gated sodium channels and binds to collapsin response mediator protein 2, is a protected *O*-methylserine [3].

Even more important than the number of peptides accepted by the FDA is the number of peptides that are in clinical phases. In 2008, 39 were in clinical phase I, 77 in phase II, 39 in phase III, and 4 in preregistration [4].

There are several reasons for this renaissance of peptides. The first one is the fact that the number of *classical small molecules* is not increasing enormously. Furthermore, several comparisons with *small molecules* are favorable to peptides. Thus, the well-defined peptide chemistry allows an easier way to prepare analogs. Pharmaceutical companies have also detected a better manpower/milestone ratio. Peptides reach clinical phases more easily. In parallel, advances in the fields of formulation and drug delivery technology, and the fact that these technologies are accepted for the introduction of a peptide into the market for the first time, have fueled this field into the drug