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Disulfide-rich macrocyclic peptides as templates in drug design

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Highlights:

- Naturally occurring cyclic disulfide-rich peptides have exceptional stability and rigid 'framework' structures
- Pharmaceutically interesting peptide epitopes can be grafted into these frameworks to constrain the bioactive structure of the epitope, thus increasing its stability and activity
- Cyclic disulfide-rich peptides are excellent frameworks for drug design and provide new prospects for investigating challenging pharmaceutical targets

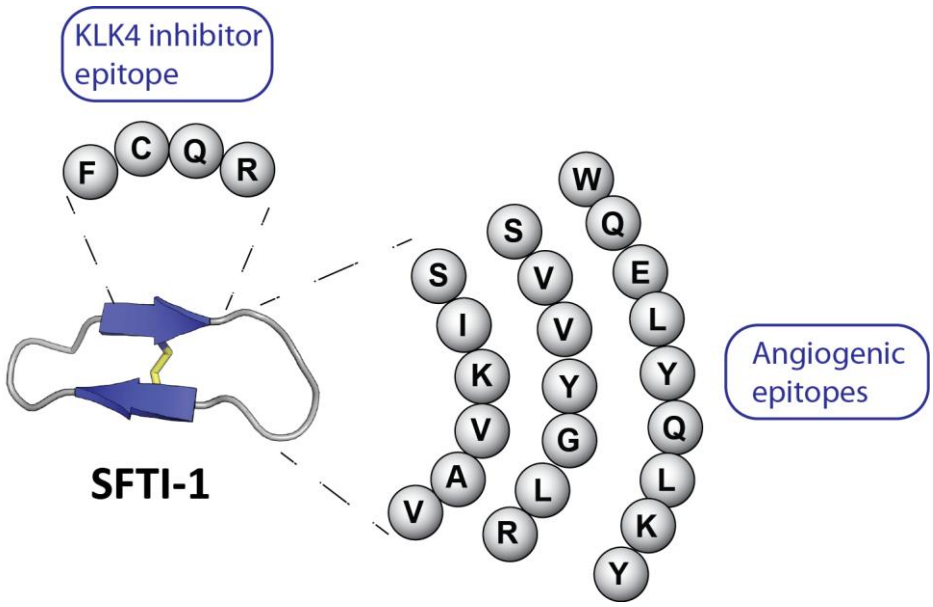
Abstract:

Disulfide-rich head-to-tail cyclic peptides are abundant in nature, ranging in size from ~12 – 40 residues. Recently they have attracted the interest of medicinal chemists owing to their exceptional thermal, chemical and enzymatic stability brought about by their constrained structures. Here we review current trends in the field of peptide-based pharmaceuticals and describe naturally occurring cyclic disulfide-rich peptide scaffolds, discussing their pharmaceutically attractive properties and benefits. We describe how we can utilise these stable frameworks to graft and/or engineer pharmaceutically interesting epitopes to increase their selectivity and bioactivity, opening up new possibilities for addressing 'difficult' pharmaceutical targets.

Key Words:

Disulfide bonds, cyclic peptide, cyclotide, grafting

Graphical Abstract:



Abbreviations:

BBI, Bowman Birk inhibitor; BTD-2, baboon θ-defensin 2; CCK, cyclic cystine knot; CsA, Cyclosporine A; GC-C, guanylyl cyclase C; GPCR, G-protein coupled receptor; HIV, human immunodeficiency virus; kB1, kalata B1; KLK4, kallikrein-related peptidase 4; LAM, laminin α1 chain; MOG, myelin oligodendrocyte glycoprotein; NME, new molecular entity; nAChR, nicotinic acetylcholine receptor; OPN, osteopontin; PPI, protein-protein interaction; QK, a VEGF peptide mimic; RTD-1, rhesus θ-defensin 1; SFTI-1, sunflower trypsin inhibitor-1; US FDA, United States Food and Drug Administration.

1. Introduction

Therapeutic molecules currently on the market can be broadly divided into two categories according to their molecular weights – small molecules of <500 Da and biologics of >5000 Da. Biologics typically have higher target potencies and specificities than small molecules due to an increased number of interactions with their targets. These favourable features mean that they potentially have fewer off-target side-effects than small molecule drugs. However, disadvantages associated with biologics include their generally poor membrane permeability, low bioavailability and metabolic instability. Thus, biologics typically require delivery via injection, which is not a preferred route of administration due to poorer patient compliance, higher cost of goods and more stringent storage requirements than small molecule drugs. These disadvantages are generally not observed with small molecule drugs, which mostly obey Lipinski's 'rule-of-five' [1]. In addition to having the favourable drug-like pharmacokinetic properties associated with this rule, small molecule drugs are also relatively inexpensive to manufacture. However, small molecules often suffer from low target specificities that can lead to side-effects. In some cases, side-effects might be harmful and not discovered until after the drug has reached the market [2].

Peptide-based drugs offer an alternative class of molecules for drug development that can tackle molecular spaces that have been considered undruggable by small molecules or biologics [3]. In this article we focus on a particular class of peptides, namely disulfide-rich peptide macrocycles, as we believe that these are an especially promising group of molecules for drug development.

In general, the field of peptide-based therapeutics has received growing attention from the pharmaceutical industry in recent years, in large part due to some of the attractive properties of peptides compared to small molecule or protein-based drugs, including their unique size range, synthetic accessibility and combinatorial diversity [3-5]. Peptide-based therapeutics include peptides comprised solely of proteinogenic amino acids, as well as peptides incorporating a variety of modifications, including cyclisation, incorporation of unnatural amino acids (eg. ß-amino acids),

alternatives to amide linkers (eg. esters) and linker groups (eg. disulfide bonds, PEG linkers). A few examples of peptide-based drugs currently on the market that include some of these modifications are shown in **Figure 1**.

The wide range of disease states that can be targeted by peptide therapeutics is exemplified by these molecules, which target the immune, gastrointestinal and hemopoetic systems, and other peptide-based drugs, including Capoten® (hypertension), Aggrastat® (thrombotic disorders), Integrilin® (acute coronary syndrome), Angiomax® (unstable angina), Prialt® (neuropathic pain) and Byetta® (type 2 diabetes) [5, 6]. This wide range of targets highlights the broad potential of peptide-based therapeutics. Nevertheless, peptides still account for only a small fraction of new drugs reaching the market: during 2011-2013, there were 96 new molecular entities (NMEs) approved by the US FDA, of which, nine were peptide therapeutics (**Table 1**) [7-9]. This review describes the current state of peptide-based therapeutics, and explores approaches to 'difficult' targets using disulfide-rich macrocyclic peptides. We conclude with a discussion of possible future trends and challenges in this field.

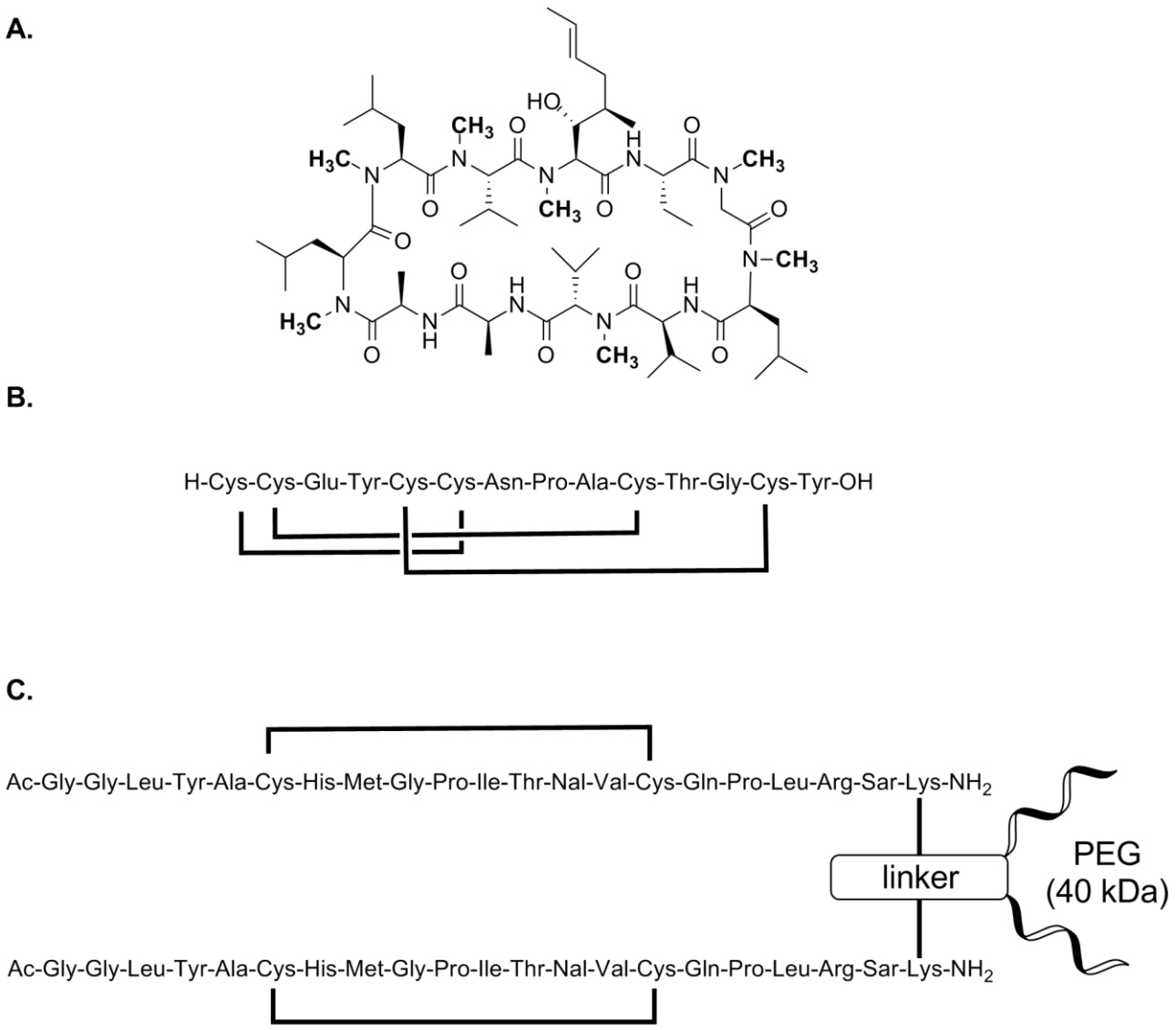


Figure 1. Structures of selected examples of peptide-based therapeutics approved by the US FDA. Disulfide bond connectivity is indicated by bold lines linking cysteine residues in panels B and C. **A.** Cyclosporin A; **B.** Linaclotide; **C.** Peginesatide (Nal = 3-(1-napthyl)alanine and Sar = N-methylglycine).

Table 1

Peptide-based therapeutics approved by the US FDA in the period 2011-2013[a]

Generic name (trade name)	Disease/Target	Properties	Structural/Therapeutic features
Boceprevir (Victrelis®)	Chronic hepatitis C infection	NS3/4A protease inhibitor	Short linear peptidomimetic; non-proteinogenic amino acids; short half-life
Telaprevir (Incivek®)	Chronic hepatitis C infection	NS3/4A protease inhibitor	6-mer linear peptide; non-proteinogenic amino acids
Icatibant (Firazyr®)	Hereditary angioedema	Bradykinin B_2 receptor antagonist	10-mer linear peptide; non-proteinogenic amino acids
Lucinactant (Surfaxin®)	Prevention of respiratory distress syndrome	Pulmonary surfactant	Mixture of active peptide (Sinapultide), lipids and fatty acid
Peginesatide (Omontys®)[b]	Anaemia due to chronic kidney disease	Synthetic, PEGylated erythropoiesis-stimulating agent	Disulfide-stabilised and PEGylated; long half-life (24-72 h)
Carfilzomib (Kypolis®)	Multiple myeloma	20S proteasome inhibitor	Linear tetrapeptide; extensive metabolism and short half-life
Linaclotide (Linzess®)	Irritable bowel syndrome with constipation; chronic idiopathic constipation	Guanylyl cyclase C agonist	Linear disulfide-stabilised 14-mer; orally administered
Pasireotide (Signifor®)	Cushing's disease	Somatostatin analogue	Cyclic hexapeptide; 12 h half-life; non-proteinogenic amino acids
Teduglutide (Gattex®)	Short bowel syndrome	Glucagon-like peptide 2 analogue	33-mer linear peptide; short half-life

[a] Listed in chronological order of approval dates. Data current as of December 2013.
[b] Drug voluntarily recalled in March 2013.

2. Peptides as drugs

Currently approved drugs target only about 2% of proteins encoded in the human genome and many of these targets are confined to specific classes of proteins such as GPCRs, enzymes, ion channels and transporters [10]. This low proportion of therapeutic targets could be greatly increased by targeting protein-protein interactions (PPIs), which are crucial in many biological processes. PPIs are difficult to target because the interaction interfaces are often composed of large and relatively flat surface regions [11, 12]. Hence, they are generally considered especially difficult to target by small molecule therapeutics. As a result, recent years have seen an explosion in the development and approval of biologics targeting these interactions. Their success stems both from their larger size and the ability to adopt defined 3D structures that drive their exquisite target specificity and selectivity, as well as from recent advances in recombinant DNA technology and heterologous expression that enables precision engineering of large and complex biomolecules.

Peptide-based therapeutics (defined here as including peptides with a MW of 500 — 5000 Da) have the potential to display the advantages of *both* small molecule therapeutics *and* biologics (**Table 2**). They are significantly smaller than biologics but often equally specific and potent. Most importantly and unlike small molecule therapeutics or biologics, they are amenable to chemical *or* genetic engineering approaches for their synthesis. A combination of these alternative synthetic approaches has proven instrumental in the development of peptide-based therapeutics. For example, peginesatide (**Figure 1C**), a synthetic erythropoiesis-stimulating agent mimicking the action of endogenous erythropoietin was identified via phage display in 1996 [13]. The identified disulfide-bridged cyclic lead peptides were then chemically optimised via: (i) dimerisation of the ligand peptide through covalent crosslinking, which yielded molecules with significantly improved potency [14]; and (ii) PEGylation, which resulted in a dramatically improved circulation half-life (24-72h) and reduced immunogenicity [15]. Peginesatide was approved by the FDA in 2012 for the treatment of anaemia associated with chronic kidney disease.

Unfortunately, but illustrative of the fact that drug development remains a risky business, even for peptide-based drugs, peginesatide was recalled in 2013 due to serious hypersensitivity reactions in 0.2% of patients [16]. Nevertheless, there remains optimism that peptide-based therapeutics are likely to be subject to fewer side effects than small molecule drugs. In part this derives from their high specificity and in part from the fact that their metabolic products are amino acid based and hence non-toxic.

Unlike the specific drawback applying to many drugs of harmful or undesired side-effects, which needs to be considered on a case-to-case basis, the previously recognised generic drawbacks of peptide-based drugs (e.g. poor serum stability, rapid elimination and low bioavailability) can be ameliorated using a vast array of modifications. Modifications that have been applied to peptide-based drugs that have progressed to the market include head-to-tail cyclisation (increased stability), N-methylation (increased membrane permeability and proteolytic stability), inclusion of non-natural amino acids (increased specificity and stability), PEGylation (reduced clearance) as well as inclusion of structural constraints such as linkers and bridges (eg. disulfide bonds). The non-ribosomally synthesised cyclic un-decapeptide cyclosporin A (CsA; **Figure 1A**) has been at the forefront of peptide-based therapeutics for many years because of its potent immunosuppressive activity and favourable pharmacology. This fungus-derived peptide incorporates many of the aforementioned modifications, including N-methylation, head-to-tail cyclisation, and non-proteinogenic amino acid building blocks, which contribute to CsA's high lipophilicity and allow it to cross biological membranes. As a result, CsA has one of the highest oral bioavailability values reported for peptides (F~30%) and can effectively engage its cytosolic target cyclophilin/calcineurin [17].

CsA highlights the potential value of peptides derived from natural sources (bacteria, fungi, plants, animal venoms) for drug discovery and development [18-20]. This value is further underscored by the recent example of linaclotide, a drug approved by the FDA in 2012 for the treatment of irritable

bowel syndrome with constipation [21]. This 14-mer peptide derived from the *Escherichia coli* enterotoxin STh, is structurally constrained by three disulfide bridges and is a potent agonist of intestinal guanylyl cyclase C (GC-C). STh is structurally related to the endogenous human GC-C agonists guanylin and uroguanylin, which lack one of the three disulfide bonds present in STh. Guanylin and uroguanylin each exist as two slowly interconverting topological isomers of which only one is able to stimulate GC-C. Because this isomerism is absent in STh, it appears that the role of the additional disulfide bond is to "lock-in" the active conformation [22]. Such constrained molecules with diminished conformational flexibility pay a reduced entropic penalty upon target engagement, typically resulting in superior binding affinities and potencies. These are attributes that are typically associated with disulfide-rich macrocycles, the focus of this review.

Table 2

Summary of drug-like pharmacokinetic properties of small molecules, peptides and biologics

	Small molecules	**Peptides**	**Biologics (proteins)**
Molecular weight (Da)	< 500	500 - 5000	> 5000
Target specificity	Generally low, but exceptions	Generally high	Generally high
Typical target	Intracellular/extracellular/ membrane bound	Mostly extracellular/membrane bound (some exceptions)	Extracellular / membrane bound
Follow Lipinski's rule	Generally, yes	No	No
Production	Chemical synthesis isolation from natural sources	Chemical synthesis, isolation from natural sources, heterologous expression	Heterologous expression, isolation from natural sources
Amenability to engineering	Relatively high (but limited by size)	High	Relatively low (limited to proteinogenic residues)
Oral bioavailability/ bioactivity	Frequent, relatively well understood	Some examples, poorly understood	No
Clearance	Variable but tuneable	Generally high, but tuneable with chemical modification	Low, difficult to tune
Stability - *in vivo* - *ex vivo*	Variable but tuneable High	Variable, but tuneable High	Low Low
Immunogenicity	Low	Potentially	Potentially
Tissue penetration	High	High	Low
Toxicity/side-effects	Relatively high	Generally low	Generally low
Metabolism	Complex	Amino acids	Amino acids

3. Disulfide-rich cyclic peptide frameworks with pharmaceutical applications

Naturally occurring disulfide-rich peptides are attractive as lead molecules for medicinal chemists owing to their remarkable stability and typically highly specific pharmacological profiles. Disulfide bonds play a role in both structure and function of peptides [23], and are particularly noted for their ability to favour constrained and well-defined 3D structures. Well-defined structures can result in increased potency [24], stability [25], selectivity [26], permeability [27], as well as reduced susceptibility to degradation by proteases [28], and an ability to lock peptides in an active conformation [29]. Mimicry and/or stabilisation of secondary structures are particularly critical considerations when designing inhibitors of PPIs for therapeutic targets [30].

Aside from the incorporation of disulfide bonds, head-to-tail cyclisation is another approach that has been adopted in nature to stabilise peptides [31-34]. The focus of recent work in our laboratory has been to examine peptides that combine this modification with disulfide bonds, as such peptides have exceptional stability and make excellent frameworks for drug development applications. In the following sections we describe examples of disulfide-rich cyclic peptide frameworks, including their structures, applications and potential in pharmaceutical development. We have focussed on examples studied by our group, based the frameworks illustrated in **Figure 2**. A number of other recent reviews have covered the topics of disulfide-rich peptides [3, 35, 36] or cyclic peptides [37-39], but we focus here on frameworks combining *both* structural elements.

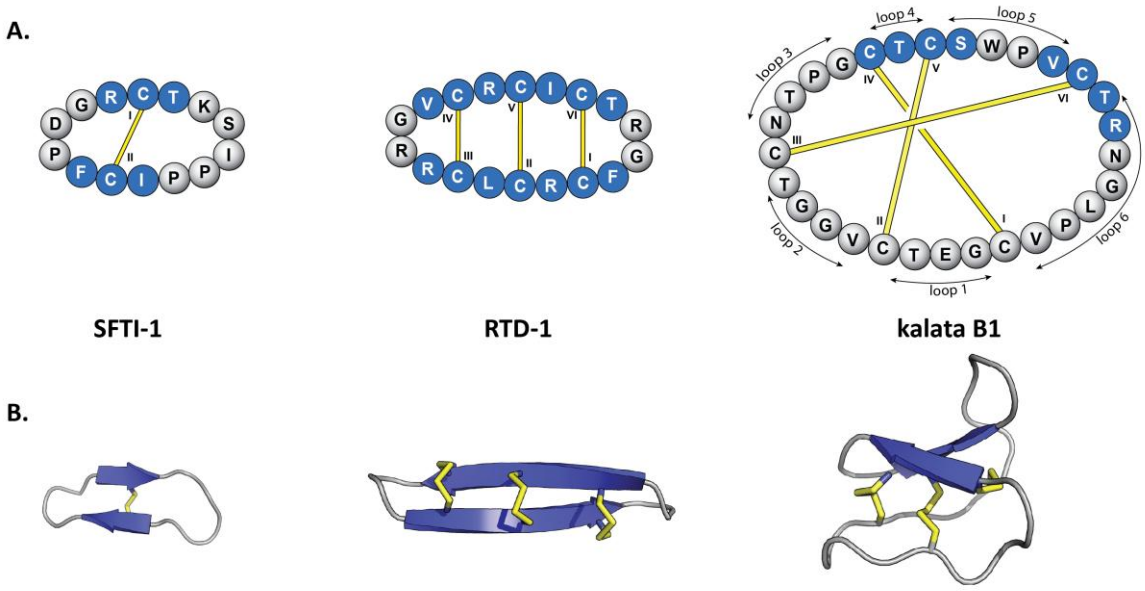


Figure 2. Sequences and structures of three disulfide-rich peptide families, represented by SFTI-1 (PDB ID: 1JBL), RTD-1 (PDB ID: 2LYF), and kalata B1 (PDB ID: 1NB1). **A.** Simplified structural diagrams of the three peptides, highlighting residues that comprise ß-sheets and disulfide bonds. The sequences are drawn in a clockwise direction for each peptide. The disulfide connectivity is displayed, with cystine residues identified using Roman numerals. **B.** Secondary structures of the three disulfide cyclic peptides, showing ß-sheets as arrows. The structure of RTD-1 has been flipped 180° in the horizontal plane to better show the disulfide connectivity.

3.1 SFTI

Sunflower trypsin inhibitor-1 (SFTI-1) is a 14-amino acid cyclic peptide composed of two short anti-parallel β-strands stabilised by a single disulfide bond and an array of hydrogen bonds [40, 41]. It belongs to the Bowman Birk Inhibitor (BBI) class of proteins, and is the smallest and most potent known peptidic inhibitor of trypsin [42]. Like other BBIs, which have been studied for their anti-cancer or anti-inflammatory properties [43], SFTI-1 has been recognised for its therapeutic potential [44]. For example, it has been shown to be a potent inhibitor of matriptase, a type II transmembrane serine protease, which promotes cancer cell metastasis through its proteolytic function [45, 46].

Both the disulfide bond and cyclic backbone of SFTI-1 contribute to its activity and stability. Specifically, the trypsin-inhibitory activity of SFTI-1 is substantially reduced if either the disulfide bond is removed [47] or the cyclic backbone is opened (i.e. between Asp^{14}-Gly^{1}) [48]. The effect of these structural perturbations on the activity of the native peptide is presumably due to the reduced rigidity and/or decreased proteolytic stability that they introduce. Indeed, removal of the disulfide bond from SFTI-1 not only weakens the hydrogen bond network [48] but renders the molecule susceptible to trypsin proteolysis [49]. SFTI-1 is therefore a natural example of how a cyclic backbone combined with a single disulfide bond can enhance the activity and stability of a peptide.

3.2 θ -Defensins

In mammals, defensins are classed into three subfamilies, referred to as the α-, ß- or θ-defensins [50, 51]. They are all characterised by six conserved cystine residues, forming three disulfide bonds (although the disulfide connectivity differs across the three subfamilies), together with ß-sheet regions and an overall net positive charge. θ-Defensins are cyclic 18 residue disulfide-rich peptides, originally isolated from the leukocytes of rhesus macaques [52] and baboons [53]. Their structural features include a head-to-tail cyclic backbone and three parallel disulfide bonds arranged in a 'cyclic cystine ladder' motif [54], with a disulfide connectivity of Cys^{I}-Cys^{VI}, Cys^{II}-Cys^{V} and Cys^{III}-Cys^{IV} (**Figure 2**). θ-Defensins have various inherent pharmaceutically-relevant activities, including antimicrobial activity [55, 56]; antiviral activity [57] (including anti-HIV activity [58]); antitoxic properties [59]; and immunomodulatory effects [60]. Their stable structure and low toxicity has made them a focus for drug development applications. Much of the published work on θ-defensins has focussed on their anti-HIV and antibacterial mechanisms of action, and they are being developed as topical microbicides [61].

The role of the cyclic cystine ladder on stability and antibacterial activity in θ-defensins has recently been examined [62]. Systematic removal of disulfide bonds in the cyclic cystine ladder of

baboon θ-defensin 2 (BTD-2) showed that the disulfide bonds played an important role for structure and stability of θ-defensins, but were not required for membrane binding or antibacterial activity. It has been suggested that the role of the disulfide bonds in these defensins is to bolster antimicrobial activity by allowing the peptides to remain stable in a diverse range of environments [62]. Overall, the plasticity, stability and multiple activities of these peptides makes them interesting therapeutic leads, and promising scaffolds for peptide drug design.

3.3 Cyclotides

Cyclotides [63] are a large family of cyclic peptides that are widely distributed throughout the plant kingdom, being identified so far in members of the Rubiaceae, Violaceae, Solanaceae, Fabaceae and Cucurbitaceae plant families [64-66]. The defining structural characteristic of cyclotides is their cyclic cystine knot (CCK) motif- a topologically more complex motif than the cyclic cystine ladder of the θ-defensins. This motif is composed of a head-to-tail cyclic backbone and a cystine knot, which comprises an embedded ring formed by two disulfide bonds and their connecting backbone segments as well as a third disulfide bond that threads through the embedded ring [63]. The intervening backbone sequences between successive cystine residues are referred to as loops and display residues that define the various bioactivities of cyclotides, which are of interest from an agrochemical and/or pharmaceutical perspective. These bioactivities include insecticidal [67], uterotonic [68], HIV inhibitory [69], antimicrobial [70], cancer cell toxicity [71], and immunosuppressant activities [72]. The natural function of cyclotides is thought to be as pesticidal agents based on their insecticidal activity. Their various pharmaceutically related activities were discovered fortuitously in a wide range of screening programs.

Aside from being of interest because of their various natural activities, cyclotides are attractive scaffolds for drug design because they are exceptionally stable. The prototypic cyclotide, kalata B1, is resistant to chaotropic agents such as 6 M guanidine hydrochloride or 8 M urea, temperatures

approaching boiling, acids, and a range of proteases, including conditions under which most proteins denature or degrade. Removal of a single disulfide bond from kalata B1 not only reduces its conformational rigidity but also renders it more susceptible to denaturation by chemical agents [73]. Complete removal of all disulfide bonds further reduces the stability of kalata B1; for example, a reduced and alkylated form of kalata B1 is degraded by trypsin within 15 minutes whereas the native form is stable for over 6 hours [73]. Although two acyclic mutants of kalata B1 showed comparable stability to the native form following incubation with trypsin, endoproteinase Glu-C and thermolysin, acyclic mutants are less stable against proteolysis by exoproteases [73]. Overall, the combination of a cyclic backbone and a knotted disulfide arrangement, both of which underpin the remarkable stability of cyclotides, together with the possibility to introduce combinatorial variation within these sequences, make cyclotides promising scaffolds for design and development of peptide-based therapeutics.

It is noteworthy that SFTI-1, the θ-defensins and the cyclotides represent a series of cyclic disulfide-rich peptides with increasing size and complexity, i.e. from a 14-mer peptide with one disulfide bond, to 18-mer peptides with three laddered disulfide bonds, to 30-mer peptides with three knotted disulfide bonds. Between (and beyond) these examples there are a variety of other disulfide-rich peptides that also offer potential as scaffolds, including some natural peptides artificially engineered to be cyclic.

3.4 Engineered cyclic disulfide-rich frameworks

In addition to using naturally occurring cyclic peptides as pharmaceutical templates, it has become increasingly popular to manipulate natural acyclic disulfide-rich peptides using cyclisation to improve on their pharmaceutical properties. This has now been done for a variety of different peptides with varying degrees of success, including for the conotoxins Vc1.1 [74] and MrIA [75], chlorotoxin [76] and hepcidin [77]. Below we describe selected examples.

3.4.1 Conotoxins

Conotoxins, peptides isolated from the venoms of carnivorous marine cone snails, target neuronal ion channels in the prey of the snails, as well as fortuitously interacting with various receptors in humans [78]. For the latter reason they have been of great interest for the development of novel therapeutics, especially in the area of pain therapies [79]. Conotoxins are disulfide-rich peptides typically between 12-40 residues in length [80] and have been divided into structural classes and pharmacological families based on their cysteine frameworks, pharmacological targets and gene superfamilies [81]. For several of the conotoxin classes, the N- and C-termini are located relatively close in space [82] and some of these peptides have successfully been cyclised by bridging the termini using linkers of varying lengths. Initially α-conotoxin MII, targeting the nicotinic acetylcholine receptor (nAChR), was cyclised by the introduction of a six-residue linker, leading to a stabilised structure with increased resistance against proteolysis, while retaining biological activity [83]. The same concept has since been applied to several other α-conotoxins, including RgIA [84], AuIB [85], and ImI [86]. Most notable was the incorporation of a six-residue linker in α-conotoxin Vc1.1, cyclising the peptide and leading to the engineering of an orally active conotoxin derivatie with potential for the treatment of neuropathic pain [74].

In addition to the cyclisation of α-conotoxins, the peptides MrIA and MVIIA, a χ-conotoxin and ω-conotoxin respectively, have been successfully cyclised. MrIA is a 13-residue peptide with four cysteine residues arranged in a ladder conformation that inhibits the human norepinephrine transporter [87]. For the last few years a close derivative of this natural peptide has been undergoing clinical trials for neuropathic pain under the name Xen2174 [88]. In an attempt to make an improved derivative, MrIA was successfully cyclised by incorporation of two additional residues linking the N- and C-termini and the cyclic derivative showed no perturbation of structure or loss of activity [75]. However, because of the already relatively high stability of acyclic Xen2174 it was

felt that the small improvement in stability brought about by cyclization did not warrant further development of cyclic MrIA. In another recent example, a 25-residue acyclic peptide isolated from *Conus magus*, MVIIA, comprising three disulfide bonds arranged in an inhibitory cystine knot motif [89] has been cyclised. Native MVIIA inhibits N-type voltage-gated calcium channels, and a synthetic version of the peptide is approved for intrathecal use to treat chronic pain [90, 91] under the generic name ziconotide and trade name Prialt® [92]. Tam and co-workers recently reported cyclisation of conotoxin MVIIA by incorporation of a 4-residue linker, GGPG [93], although the paper provided no structural or activity data to describe any advantages of cyclising the peptide. It will be of interest to see whether the cyclic molecule provides benefits over the marketed acyclic peptide.

3.4.2 Chlorotoxin

Chlorotoxin is a 36-residue peptide containing four disulfide bonds that was first isolated in 1993 from the giant Israeli scorpion *Leiurus quinquestriatus*. It was named based on its ability to inhibit chloride-channels [94] but was subsequently found to bind to malignant glioma cells [95, 96], with cell surface matrix metalloproteinease-2 [97] and annexin A2 [98], rather than chloride channels identified as two molecular targets. Owing to the preference of chlorotoxin for tumour cells, the peptide was labelled with the dye Cy5.5 (CTX:Cy5.5) and is being developed as “tumour paint” to be used during surgery as an optical imaging contrast agent to allow surgeons to distinguish between healthy and cancerous tissues [99]. Although chlorotoxin is relatively stable, due to its four disulfide bonds, a cyclic version of the peptide was engineered by addition of a seven-residue linker (GAGAAGG) to bridge the N- and the C-termini [76] in an attempt to further improve stability. Cyclic chlorotoxin was found to retain the native three-dimensional structure in solution of the wild-type peptide and showed improved structural stability in serum, with a 15-20% increase in resistance to degradation over its linear counterpart [76]. A combination of stabilisation technology

using cyclisation with other approaches for the therapeutic targeting of chlorotoxin make the peptide of ongoing interest. For example, a recent study showed that TM601, a synthetic version of chlorotoxin, uses clathrin-mediated entry to internalise into cells and that the toxin's localisation pattern differs in glioma cells compared to normal cells [100], making chlorotoxin an exciting molecule for potentially delivering cargo for cancer therapeutics. Chlorotoxin is currently being coupled to liposomes encapsulating antisense oligonucleotides or small interfering RNA, and preferentially delivers its cargo to glioma cells over non-cancer cells [101].

3.4.3 Hepcidin

Another example where a disulfide-rich peptide has been cyclised in an attempt to improve biopharmaceutical properties is hepcidin, a key regulatory hormone for iron homeostasis in vertebrates [102]. This 25-residue peptide with four disulfide bonds linking Cys^{I}-Cys^{VIII}, Cys^{II}-Cys^{IV}, Cys^{III}-Cys^{VI} and Cys^{V}-Cys^{VII} [103] has a flexible N-terminus that is crucial for the interaction between hepcidin and its receptor ferroportin [104]. Hepcidin is deactivated *in vivo* by cleavage of the flexible N-terminal, and it was therefore believed that head-to-tail cyclisation might improve stability and lead to more attractive hepcidin drug leads [77]. Four backbone cyclic analogues of hepcidin were examined for structural perturbations and alterations in activity profile. Although cyclisation was successful and three of the four analogues showed similar secondary Hα chemical shift profiles to the linear peptide, all cyclic analogues were found to be inactive [77], most probably due to unfavourable conformational restriction of the flexible N-terminal. This result highlights the importance of structural considerations when introducing stabilising elements into peptides. In the case of hepcidin, the lesson learned is that if the peptide termini are important for activity, then cyclization is not a feasible option.

4. Engineering disulfide-rich macrocyclic peptides for 'difficult' pharmaceutical targets

Disulfide-rich macrocyclic frameworks such as those presented in the previous section are of interest for pharmaceutical applications not only for their inherent pharmacological actions (eg. antibacterial or antiviral activities) but for their exceptional stability. Thus, an alternative approach to focusing on the inherent activity of a framework is to introduce a desired new activity into the framework while maintaining the intrinsic stability of the framework. The chemical approach that we employ to transfer the activity of a peptide epitope into a framework is called grafting (**Figure 3**) and has been widely applied over the last few years [66, 105-109]. Grafting small, bioactive epitopes into highly stable frameworks has the potential to increase both stability and bioactivity of the peptide epitope, which would otherwise have poor *in vivo* stability. Sequestration of an active peptide into a rigid template can have other potential benefits, including constraint of an active conformation, thereby reducing the entropy cost of binding of the peptide to a specific target, and improvement of the overall pharmacokinetic properties. Here we describe examples of grafting therapeutically relevant sequences into naturally occurring disulfide-rich cyclic peptides. We additionally discuss engineered peptides, in which disulfide bonds have been introduced to peptide epitopes with the intent of inducing a desired stable secondary structure to stabilise the peptide pharmacophore. Both approaches offer promise in addressing targets previously considered undruggable.

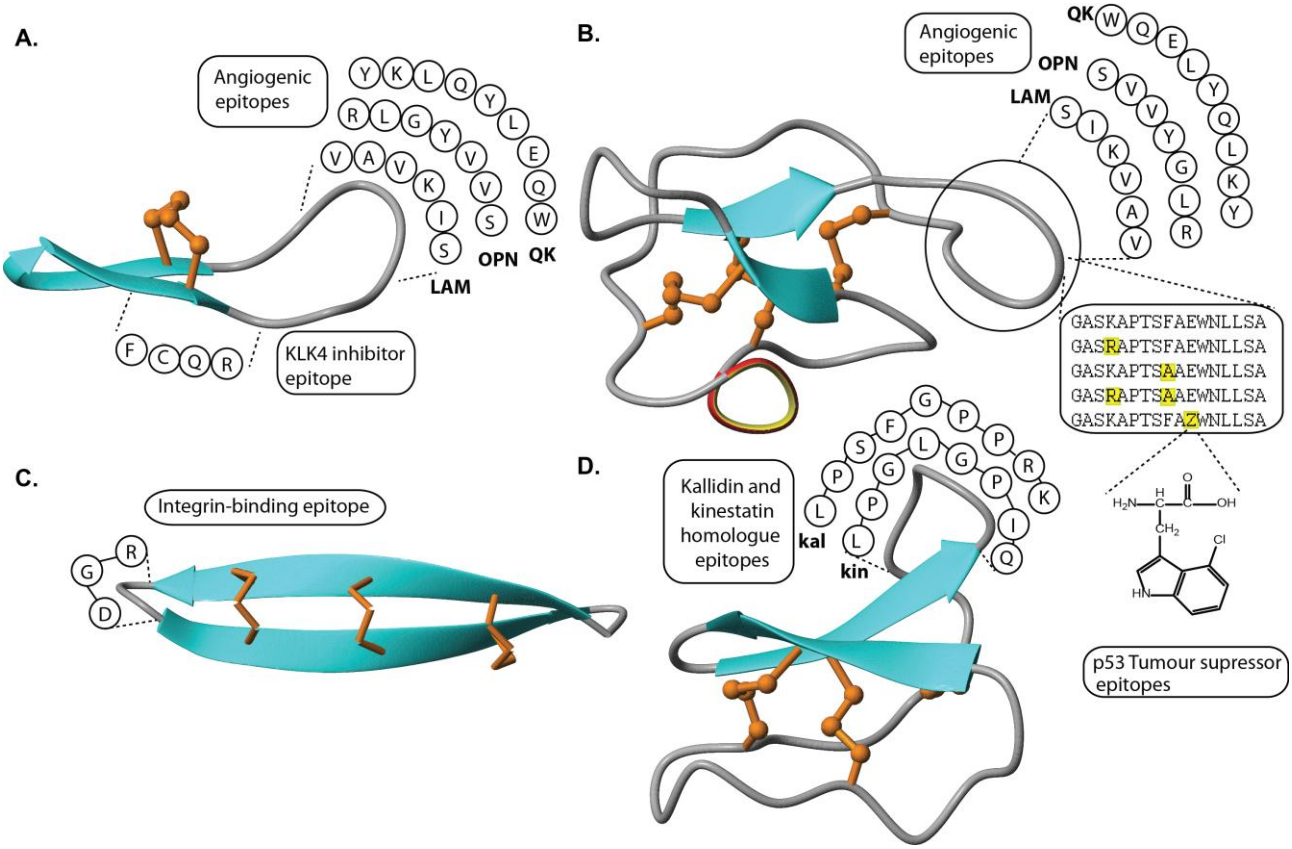


Figure 3. Grafting pharmaceutically-relevant peptide epitopes onto disulfide-rich cyclic peptides. **A.** Angiogenic epitopes from laminin α1 chain (LAM), osteopontin (OPN) and a VEGF peptide mimic (QK) have been grafted into SFTI-1 (PDB ID: 1BJL) [105]. Another example of successful peptide epitope grafting into SFTI-1 is the introduction of the KLK4 inhibitor epitope to produce a peptide that was active against kallikrein-related peptidase 4, which is up-regulated in prostate cancer [108]. **B.** The same pro-angiogenic epitopes have been grafted into loop 6 of MCoTI-II (PDB ID: 1HA9) to create stable pro-angiogenic peptides [105]. A series of p53 tumour suppressor peptide epitopes have been grafted into the same loop by Camarero and co-workers to create a cyclic stable peptide that activates the p53 tumour suppressor pathway (modified residues indicated by shading) [107]. **C.** The cyclic cystine ladder peptide θ-defensin RTD-1 (PDB ID: 2LYF) was used to graft the well-known integrin binding epitope 'RGD' and showed activity against $\alpha_v\beta_3$ integrin [106]. **D.** Two epitopes derived from kallidin and kinestatin were grafted into loop 6 of kalata B1 (PDB ID: 1NB1) to produce a cyclotide with potential for the treatment of inflammatory pain [110].

4.1 SFTI grafting

Human kallikrein-related peptidase 4 (KLK4) is a trypsin-like serine protease that is a potential target for prostate cancer treatment and is weakly inhibited by SFTI-1. Using a combined substrate library screening and computer-guided design approach, SFTI-1 was re-engineered to selectively target KLK4 [108, 111]. This work was recently reviewed in reference [112]. Briefly, a preferred KLK4 substrate sequence FVQR was grafted into the SFTI scaffold peptide as SFTI-FCQR (with Val substituted by Cys to maintain the critical disulfide bond required for structural stability) (**Figure 3**). This grafted inhibitor showed both potency and selectivity for KLK4 and maintained a half-life of four days in cell culture [108]. A second generation derivative of SFTI-FCQR (D14N) with an *in silico* optimized internal hydrogen bond network showed exquisite selectivity over closely related serine proteases, and was detectable in serum after interperitoneal administration to mice [111].

SFTI-1 has also been used as a grafting framework in the design of pro-angiogenic peptides [105]. Pro-angiogenic agents have therapeutic potential in multiple disease states, including cardiac ischemia, wound healing, and rheumatoid arthritis [113, 114]. In a similar approach to the aforementioned KLK4 inhibitors, SFTI-1 was grafted with three epitopes that have been reported to promote potent angiogenic activity. This set of peptides comprised a hexapeptide from the laminin α1 chain (LAM, residues 2105-2110) [115], a heptapeptide from osteopontin (OPN, residues 162-168) [116], and a VEGF mimic (QK, residues 17-25) [117]. The small size of these peptides made them ideal candidates for grafting into the SFTI-1 framework, and as part of the same study they were also grafted into loop 6 of the cyclotide MCoTI-II. The peptide sequences were shown to have adopted conformations in the constrained SFTI-1 framework that allowed for optimal binding interactions with receptors on endothelial cells. The grafted peptides also showed an increase in angiogenic activity and stability in plasma over the original linear epitopes. These examples serve

to highlight that SFTI-1 is a very suitable scaffold for peptide grafting, and for improving the stability and bioactivity of the grafted epitopes.

4.2 θ –Defensin grafting

The highly constrained cyclic cystine ladder of θ-defensins affords a valuable framework for peptide grafting, offering excellent structural and chemical stability. The ladder is comprised of two antiparallel β-strands, and the spacing of the cystine residues results in the disulfide bonds being co-located on one face of the molecule (**Figure 2**). Although grafting in the turn region of the framework is limited to four residues (unless the loop size is expanded), the extended β-sheet region could be used for grafting peptide epitopes that target β-sheet PPIs. Structural investigations of the role of the cyclic cystine ladder have revealed that one or two disulfide bonds can be removed without compromising the structure and stability of the peptide, thereby providing additional space for grafting [62]. Recently this framework has been investigated for grafting applications by the introduction of the integrin-binding Arg-Gly-Asp (RGD) motif into either one or both loops of rhesus θ-defensin 1 (RTD-1) (**Figure 3**) [106]. Of the series of grafted analogues produced in this study, the most active had an IC_{50} of 18 nM for the $\alpha_V ß_3$ integrin receptor and had high serum stability relative to the parent peptide RTD-1. Successful incorporation of two RGD motifs in one θ-defensin scaffold demonstrated that the cystine ladder additionally offers the possibility of designing grafted peptides with two bioactive epitopes, thus potentially offering increased activity or dual specificity.

4.3 Cyclotide grafting

The potential of cyclotides as molecular scaffolds has been demonstrated by many successful examples of modified cyclotides directed against a range of targets, including pro-angiogenesis

pathways [105], the p53 tumour suppressor pathway [107], bradykinin B_1 receptor [110], chemokine receptor type 4 [118], neuropilin-1 and -2 [119], melanocortin 4 receptor [120], vascular endothelial growth factor-A [121], human mast cell tryptase beta [122], components of the adaptive immune system [109], thrombin [123], and integrin $\alpha_V ß_6$ [124]. Cyclotide molecular scaffolds have also been applied towards animal-based diseases, including foot-and-mouth-disease virus [125]. Although small molecule therapeutics have been proposed for some of these targets, the grafted cyclotides that have been reported collectively show that biologically active peptides of different sequence length and composition can be grafted onto a cyclotide scaffold. Here, we focus on three studies of cyclotide grafting to highlight the potential of this scaffold in the design of therapeutic compounds against difficult targets.

Multiple sclerosis is an inflammatory disorder of the central nervous system, and the immune system plays a significant role in the pathogenesis of the disease. Currently available treatments generally engage non-specific mechanisms of immune suppression, which is undesirable because the immune system is unable to respond to foreign pathogens. Antigen-specific strategies using peptides is a promising alternative in terms of properly modulating the selectivity of the immune system; however, as noted earlier in this article, the therapeutic potential of peptides is limited by their poor *in vivo* stability. In a recent study, we grafted peptide fragments from myelin oligodendrocyte glycoprotein (MOG), which possess promising immuno-regulatory potential, onto the kalata B1 scaffold (loop 5) in order to engineer stable peptide therapeutics for the treatment of multiple sclerosis [109]. We showed that the grafted peptides were resistant to enzymatic degradation and acid hydrolysis, whereas the isolated peptide epitopes degraded rapidly. Furthermore, nuclear magnetic resonance was used to confirm that the grafted peptides maintained a similar overall fold compared to the template. Evaluation of the structure of the grafted peptides is important because the structural features of cyclotides, namely the cyclic backbone and cystine knot, underpin their exceptional stability. One of the grafted peptides, MOG3, was able to significantly reduce both clinical and histological signs of multiple sclerosis in a mouse model, suggesting that

grafted cyclotides have potential as stable vaccines that can be administered intravenously to treat a number of complex immune-mediated diseases.

Peptide antagonists of the bradykinin B_1 receptor have been proposed for the treatment of chronic and inflammatory pain [126]. However, no peptidic B_1 receptor antagonists have been approved for clinical use, partly because developing such a therapeutic in a long-lived and orally active form remains a major challenge. Tam and co-workers used molecular grafting of bioactive peptides onto the kalata B1 framework to develop an orally active bradykinin B_1 receptor antagonist for inflammatory pain treatment [110]. Two grafted peptides, ckb-kal and ckb-kin, were shown to bind to the B_1 receptor using a cell-based competitive binding assay and dampen the response of the receptor to phopholipase C activation. Interestingly, the grafted peptides were able to elicit an analgesic effect when administered orally to mice, suggesting that the grafted peptides were possibly stable in the gastrointestinal tract. Compared to these cyclic analogues, a linear analogue as well as the epitope peptide itself showed poor or no activity, respectively, suggesting that the combination of cyclisation and the cystine knot were important for the stronger oral activity of the grafted cyclic peptides.

In work by Camarero and co-workers, grafted cyclotides were used to antagonise the interaction between the transcription factor p53 and the p53 binding domain of the oncogenic proteins Hdm2 or HdmX [107]. Disruption of this interaction restores the regulatory role of p53 in protecting cells from malignant transformation. Using a murine xenograft model, the cyclotide MCoTI-II grafted with a 15-residue fragment from p53 was able to suppress tumour growth. Solution of the structure of this grafted cyclotide, MCo-PMI, complexed to the p53-binding domain of Hdm2 showed that the side chains of three residues from MCo-PMI interact specifically with the surface of Hdm2, suggesting that this interaction is responsible for the observed tumour suppression activity. In addition to demonstrating that a grafted cyclotide can modulate PPIs, this work is also noteworthy because it demonstrates that a grafted cyclotide can target an intracellular protein. This observation

suggests that molecular grafting can be used to take advantage not only of the high stability of the cyclotide framework but also of other biopharmaceutically important properties of specific cyclotides, such as cell penetration. This is a very exciting development because a large number of intracellular targets which are 'undruggable' by biologics can possibly be accessed through molecular grafting of cell penetrating cyclotides.

4.4 Applying disulfide bonds as turn mimetics

So far we have discussed the utility of disulfide-rich macrocyclic peptides as templates for grafting pharmaceutically interesting epitopes. However, disulfide bonds are also valuable as turn-inducing constraints. Recently, a combinatorial library of peptidomimetics based on the conotoxin MrIA was described. This 13-mer conotoxin contains the sequence GYKL, which is essential for biological activity [127]. Peptides in this study were designed incorporating the YKL sequence into a head-to-tail cyclic peptide, flanked on either side by various residues and two adjacent cysteine residues. Conformational flexibility was restricted by oxidising the cysteines to form a vicinal disulfide bond. The cyclic heptapeptide CCGYKLG, constrained by a vicinal disulfide bond, not only retained activity relative to the native MrIA, but also showed improved serum stability compared to the clinical candidate of MrIA, Xen2174 [128]. Interestingly, it appears that the unusual redox potential (-313 mV) of the vicinal disulfide bond contributes to the stability of the peptide, with this transoid vicinal disulfide bond showing increased stability compared to naturally occurring disulfide bonds [129]. This work further illustrates the impact that disulfide bonds and cyclisation as conformational constraints brings to the field of development of peptide drugs.

5. Conclusions

The current interest in the use of peptides as drug leads is gaining momentum and with many peptide-based drugs now on the market there are increasing opportunities to learn more about their benefits compared to small molecules or biologics. A number of difficult challenges remain in the field of peptide-based drug design, including addressing their generally low stability and oral bioavailability. Our approach to address these issues is to utilise stable naturally occurring peptide frameworks that combine a head-to-tail cyclic backbone with disulfide bonds. Several groups have now demonstrated that bioactive epitopes can be grafted into such frameworks and display selective therapeutical activities in animal models of disease. Although none have yet been proposed for human clinical trials, the approach seems to have reached proof-of-concept stage and we would be confident of further advances in this field in the near future.

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