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Using backbone-cyclized Cys-rich polypeptides as molecular scaffolds to target protein-protein interactions

Dipankar Chaudhuri¹, Teshome Aboye¹, Julio A. Camarero^{1,2}

¹Department of Pharmacology and Pharmaceutical Sciences, University of Southern California, Los Angeles, California 90089-9121, USA,

²Department of Chemistry, University of Southern California, Los Angeles, California 90089-9121, USA,

Abstract

The use of disulfide-rich backbone cyclized polypeptides as molecular scaffolds to design a new generation of bioimaging tools and drugs that are potent and specific, and thus might have fewer side effects than traditional small molecule drugs is gaining increasing interest among the scientific but also in the pharmaceutical industry. Highly constrained macrocyclic polypeptides are exceptionally more stable to chemical, thermal and biological degradation and show better biological activity when compared to their linear counterparts. Many of these relatively new scaffolds have been also found to be highly tolerant to sequence variability, aside from the conserved residues forming the disulfide bonds, able to cross cellular membranes and modulate intracellular protein-protein interactions both *in vitro* and *in vivo*. These properties make them ideal tools for many biotechnological applications. This article provides an overview of the new developments on the use of several disulfide-rich backbone cyclized polypeptides including cyclotides, θ -defensins and sunflower trypsin inhibitor peptides in the development of novel bioimaging reagents and therapeutic leads.

Keywords

cyclotides; CCK; cystine-knot; drug design; backbone cyclized polypeptides; protein-protein interactions; cyclic peptides

Introduction

The genomic and proteomic revolutions have provided us with an ever-increasing number of mechanistic insights into human diseases. Mutated genes and pathologic protein products are emerging as the basis for the development of novel therapeutic agents to treat human diseases such as cancer or autoimmune diseases. However, the selective disruption of protein-protein interactions (PPIs) still remains a very challenging task, as the interacting surfaces are relatively large and relatively flat. In fact, among the most intractable of targets

are those involving intracellular protein-protein interactions, which require the therapeutic agent to efficiently cross the cell membrane.

Broadly speaking, there are only two major structural classes of approved drugs, small molecules and protein therapeutics (also known as biologics). Small molecules typically show good stability and good pharmacological properties, but their intrinsic small size (100 atoms) endows them with only a modest overall surface area available to contact a protein target. This limits their ability to effectively target large surfaces involved in protein-protein interactions. Protein-based therapeutics, on the other hand, possess high specificity/selectivity and high affinity for protein targets (1). The use of therapeutic monoclonal antibodies to target extracellular protein receptors is just one example (2, 3). Antibodies, however, suffer from clear limitations: they are expensive to produce, cannot be delivered orally, show low tissue penetration and are unable to reach intracellular targets (4). The potential problems associated with the use of antibody fragments have led to the exploration of alternative protein scaffolds as a source for novel protein-based therapeutics (5–12). However, the utility of protein-based therapeutics has been typically hampered by their generally poor stability and limited bioavailability (13). In order to overcome these limitations, special attention has been recently given to the use of highly constrained peptides, also known as micro-proteins, for the modulation of protein-protein interactions (14–16).

In this review, we will present the recent developments in the use of Cys-rich backbone-cyclized polypeptides for targeting protein-protein interactions. Circular Cys-rich peptides are widely distributed natural product polypeptides among different species including animals and plants (Scheme 1). They have attractive features including thermal, chemical and biological stability against proteases. Peptides in this group include cyclotides, mammalian θ -defensins, and sunflower protease inhibitors.

Cyclotides

Cyclotides are fascinating micro-proteins (≈ 30 residues long) present in plants from the *Violaceae*, *Rubiaceae*, *Cucurbitaceae*, and more recently *Fabaceae* families (17). They display various biological properties such as protease inhibitory, anti-microbial, insecticidal, cytotoxic, anti-HIV, and hormone-like activities (see references (14, 15) for recent reviews of the biotechnological applications of cyclotides). They share a unique head-to-tail circular Cys-knotted (CCK) topology of three disulfide bridges, with one disulfide penetrating through a macrocycle formed by the two other disulfides and inter-connecting peptide backbones, forming what is called a cystine knot topology (Fig. 1). Cyclotides can be considered as natural combinatorial peptide libraries structurally constrained by the cystine-knot scaffold and head-to-tail cyclization but in which hypermutation of essentially all residues is permitted with the exception of the strictly conserved cysteines that comprise the knot (18–20). The main features of cyclotides are a remarkable stability due to the cystine knot, a small size making them readily accessible to chemical synthesis, and an excellent tolerance to sequence variations. For example, the first cyclotide to be discovered, kalata B1, is an orally effective uterotonic (21), and other cyclotides have been also shown to be orally bioavailable and capable of crossing cell membranes (22, 23) to efficiently target

extracellular (24–26) and intracellular PPIs *in vivo* (27). In addition, cyclotides have been shown to be poorly immunogenic due to their highly constrained nature (7, 28). Cyclotides thus appear as highly promising leads or frameworks for peptide drug design (15, 29–31).

Cyclotides are classified into three subfamilies known as the Möbius, bracelet, and trypsin inhibitor cyclotide subfamilies. All the subfamilies have the same CCK topology although the composition of the loops is slightly different (Fig. 1). Bracelet cyclotides are usually larger and more structurally diverse than Möbius cyclotides, and they make up around 60% of all the cyclotides known so far (32). Bracelet cyclotides are more difficult to obtain by chemical synthesis than either Möbius or trypsin inhibitor cyclotides due to the difficulties associated to fold them correctly *in vitro*. Due to this, bracelet cyclotides have been less used as molecular scaffolds to target PPIs.

Cyclotides from the trypsin inhibitor subfamily are found in the seeds from several plants of *Momordica* genus (33, 34) and as their name indicates are potent trypsin inhibitors (35). This is the cyclotide subfamily with fewer members identified thus far showing little sequence homology with the other cyclotides beyond the presence of the CCK fold. In fact, these cyclotides seem related to linear cystine-knot squash trypsin inhibitors and sometimes are also referred to as cyclic knottins (36). Cyclotides from this family possess a longer sequence in loop 1, making the Cys-knot slightly less rigid than in cyclotides from the other two subfamilies.

Naturally occurring cyclotides are produced in plants from dedicated genes that can encode multiple copies of the same cyclotide or even mixtures of different cyclotide sequences (37). Recent studies indicate that an asparaginyl endopeptidase (AEP)-like ligase is responsible for the backbone-cyclization of cyclotides, however, the complete mechanism of how the precursor cyclotide protein is processed has not completely elucidated yet (29). Cyclotides can also be chemically synthesized using solid-phase peptide synthesis as they are relatively small polypeptides containing typically around 30–40 residues (38). More recently, the use of protein splicing in *cis* and *trans* has also allowed the production of fully folded cyclotides inside bacterial and yeast cells using heterologous expression systems (39, 40).

Table 1 summarizes recent examples on the use of the cyclotide scaffold to target PPIs. The first two examples demonstrated the pharmacological potential of engineered cyclotides were aimed for the development of novel peptide-based novel anti-cancer (41, 42) and anti-viral peptide-based therapeutics (43). The development of molecules targeting angiogenesis has been shown to potential therapeutic avenues in cancer treatment as tumor growth is usually associated with unregulated angiogenesis. The molecular grafting of an Arg-rich peptide antagonist of the vascular endothelial growth factor A (VEGF-A) receptor onto several loops of the Möbius cyclotide kalata B1 yielded antagonists with low μM activity for blocking VEGF activity (41). This was one of the first examples where a bioactive peptide was used to produce a novel cyclotide with a specific biological activity, however, it should be noted that the biological activity would still need to be improved by several orders of magnitude for a potential pharmacological application *in vivo*. A similar approach using the molecular framework of kalata B1 was recently used in the development of cyclotides able to modulate the bradykinin and melanocortin 4 receptors for pain and obesity management,

respectively (44, 45). It is worth mentioning, that one of the designed kalata B1-based bradykinin antagonists in this work was shown to be orally bioavailable (45). In this context, a recent study using A point mutated kalata B1 cyclotide was also reported to have oral bioavailability and in a mouse model of multiple sclerosis (46). These findings highlight the potential of the cyclotide molecular framework for the development of novel orally-bioavailable peptide-based therapeutics.

The cyclotides from the trypsin inhibitory subfamily have also used as templates to engineer cyclotides with novel biological activities by means of molecular grafting. For example, the cyclotide MCoTI-I has been recently used for the design of a potent CXCR4 antagonist (47). Overexpression of the CXCR4 receptor has been observed in multiple cancers where it is believed it promotes metastasis, angiogenesis, and tumor growth and/or survival (48).

Cyclotides from the trypsin inhibitor subfamily have been also used for the development of protease inhibitors with pharmacological relevance. The cyclotide MCoTI-II was transformed into a potent and selective foot-and-mouth-disease (FMDV) 3C protease inhibitor by introducing mutations onto loops 1 and 6 (43). A similar approach was used by the same authors to generate β -tryptase and human leukocyte elastase inhibitors with low nM K_i values (42, 49).

The most exciting feature of the cyclotide scaffold is that some members of the trypsin inhibitor subfamily can cross the cellular membrane of mammalian cells, therefore, making possible the intracellular delivery of biologically active cyclotides to target intracellular PPIs (22, 23). Our group was recently able to generate a potent activator of p53 function by inhibiting the interaction between p53 and the proteins Hdm2/HdmX using the cyclotide MCoTI-I as molecular scaffold (Fig. 2) (27). The resulting cyclotide MCo-PMI was able to bind with high affinity the p53-binding domains of both Hdm2 and HdmX, showed high *ex-vivo* stability in serum and was cytotoxic to wild-type p53 cancer cell lines by activating the p53 tumor suppressor pathway both *in vitro* and *in vivo* (Fig. 2) (27). This work represents the first example showing an engineered cyclotide able to target an intracellular protein-protein interaction in an animal model of prostate cancer, therefore, highlighting the therapeutic potential of MCoTI-cyclotides for targeting intracellular protein-protein interactions. Exactly the same approach but using cyclotide MCoTI-II instead has been also employed to obtain an antagonist for the SET protein that is overexpressed in some human cancers (50).

Our group has also recently shown that cyclotides can be obtained by heterologous expression using both prokaryotic and eukaryotic expression systems (39, 40). For example, a novel MCoTI-grafted cyclotide (MCoCP4) was able to inhibit α -synuclein-induced cytotoxicity in yeast *Saccharomyces cerevisiae* (40). α -Synuclein is a small lipid-binding protein that is prone to misfolding and aggregation and has been linked to Parkinson's disease making it a validated therapeutic target for Parkinson's disease.

The ability to produce natively folded cyclotides in the cell (40, 51, 52) as described earlier makes possible the generation and rapid screening of large libraries of cyclotides, potentially containing billions of members, that are genetically-encoded cyclotides. The generation of

such tremendous molecular diversity permits the development of selection of strategies that mimic the evolutionary processes found in nature for the selection of novel cyclotide sequences able to target specific molecular targets. For example as proof of principle, our group used in cell expression of a small library based in cyclotide MCoTI-II in *E. coli* where every residue in loops 1, 2, 3, 4 and 5 was mutated to explore the effects on folding and trypsin binding activity of the resulting mutants (52). This early study revealed that most of the mutations did not affect folding on the resulting cyclotides, therefore, emphasizing the high plasticity and sequence tolerance of MCoTI-based cyclotides (52).

The use of an acyclic version of cyclotide kalata B1 was employed for the screening and selection of novel cyclotides specific for the VEGFA binding site on neuropilin-1 (53). This study used bacterial display libraries and the authors were able to obtain kalata-based cyclotides with high affinity ($K_d \approx 50$ nM), increased protease resistance, and conferred improved potency for inhibiting endothelial cell migration in vitro ($EC_{50} \approx 100$ nM) (53).

A yeast surface display approach of an acyclic version of cyclotide MCoTI-II was also employed for the screening of a linearized cyclotide library to select strong binders cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), an inhibitory receptor expressed by T lymphocytes, that has emerged as a target for the treatment of metastatic melanoma (54).

More recently, a cyclotide-based library was employed for phenotypic screening in eukaryotic cells (40). In this work, an engineered cyclotide (MCoCP4) that was designed to reduce the toxicity of human α -synuclein in live yeast cells was selected by phenotypic screening from cells transformed with a mixture of plasmids encoding MCoCP4 and inactive cyclotide MCoTI-I in a ratio of 1 to 50,000. These results show the potential for performing rapid phenotypic screening of genetically encoded cyclotide-based libraries in eukaryotic cells for the selection of bioactive compounds. These exciting results demonstrate the potential to perform phenotypic screening of genetically encoded cyclotide-based libraries in eukaryotic cells for the rapid selection of novel bioactive cyclotides. Moreover, expression in eukaryotic systems should allow the production of cyclotides with different post-translational modifications not available in bacterial expression systems.

The development of efficient methods for the chemical synthesis, cyclization and folding of cyclotides has also made possible to perform high throughput screening on chemically-generated libraries of cyclotides (26). A small library of MCoTI-based CXCR4 cyclotide antagonists was chemically obtained using a 'tea-bag' approach in combination with high efficiency folding protocols (26). The approach described in this work also included an efficient purification procedure to rapidly remove non-folded or partially folded cyclotides from the cyclization-folding crude. This approach can be also employed for the purification of cyclotide mixtures thereby allowing the synthesis of amino acid and positional scanning libraries to perform efficient screening of large chemical-generated libraries (55).

The potential of bioactive cyclotide to be employed as bioimaging agents has also been recently reported (24). An MCoTI-based CXCR4 antagonist cyclotide (MCo-CVX-6D) was shown to be an excellent bioimaging tool to visualize CXCR4-overexpressing cancer cells in a mouse model. A [^{64}Cu]-DOTA-labeled version of cyclotide MCo-CVX-6D was used for

the efficient detection of tumors containing CXCR4-expressing cells in mice using positron emission tomography-computed tomography (PET-CT) (Fig. 3).(24)

Given the good pharmacological properties of some bioactive cyclotides, their biodistribution of has been recently studied (24, 56). These studies indicate that MCoTI-cyclotides are distributed predominantly to the serum and kidneys, confirming that they are stable in serum and suggesting that they are eliminated from the blood through renal clearance (Fig. 3B) (24, 56). However, no significant uptake into the brain was observed for cyclotide MCoTI-II (56).

Mammalian θ -defensins

Defensins are cysteine-rich antimicrobial peptides (AMP) that play a critical role in the innate immune defense of mammals (57–59). Although they are classically known for their antimicrobial activities they have been also shown to be involved in other defense mechanisms including immune modulation, neutralization of endotoxins, and anti-cancer properties (59, 60). Mammalian defensins are peptides containing mostly β -sheet structures, six Cys residues forming three intramolecular disulfides and a high content on positively charged residues. They have been classified into α -, β - and θ -defensins depending on their overall structure.

In contrast with α - and β -defensins, θ -defensins are backbone cyclized peptides formed by the head-to-tail covalent assembly of two nonapeptides derived from α -defensin related precursors (Fig. 4A) (57, 61). θ -Defensins are to date the only known cyclic polypeptides expressed in animals (57).

θ -Defensins have shown to possess antimicrobial activity against both *Gram*-positive and *Gram*-negative bacteria (57), as well as anti-fungal (57) and anti-HIV (62, 63) activities.

Chemically-produced human θ -defensin, derived from human pseudogene sequences, have also shown to protect human cells from infection by HIV-1 (62) and have been even evaluated as a topical anti-HIV agent (64–66). θ -defensins have been shown to have moderate activity against several bacterial toxins and proteases (67), as well as human metalloproteases like TNF α converting enzyme (TACE) (68). θ -defensins have also been shown to possess anti-inflammatory properties in animal models (69). In addition, θ -defensins present high resistance to proteolytic degradation in serum and plasma due to the network of disulfide bonds and backbone-cyclized topology (68, 69). Altogether, these unique features have made θ -defensin an ideal molecular framework for the development of novel peptide-based therapeutics (69, 70).

Our group has developed efficient approaches for the chemical and recombinant production of θ -defensins (61, 68, 71–73). Using a ‘tea-bag’ approach in combination with a one-pot cyclization and oxidative folding method our group has obtained potent θ -defensins analog able to inhibit anthrax lethal factor (LF) and TACE with K_i values \approx 40 nM and \approx 157 nM, respectively (68). It is worth mentioning that these analogs showed also significant activity in the presence of 0.1% bovine serum albumin (BSA) (68).

The θ -defensin scaffold has also recently employed for designing integrin antagonists (74). This was accomplished by grafting the integrin-binding Arg-Gly-Asp (RGD) peptide motif into the θ -defensin RTD-1 molecular framework. The most active compound had an IC_{50} of ≈ 18 nM for the $\alpha_v\beta_3$ integrin and presented high serum stability. These results clearly show the high robustness of the θ -defensin scaffold for the generation of molecular diversity and can provide a stable and conformationally restrained scaffold for bioactive epitopes in a β -strand or turn conformation.

Sunflower trypsin inhibitor 1 (SFTI-1)

SFTI-1 is a 14 amino acid backbone-cyclized peptide containing a single disulfide bond that is naturally found in the seeds of sunflower (*Helianthus annuus*) (75). SFTI-1 belongs to the Bowman-Birk inhibitor (BBI) family, whose members are found in many plants and are potent serine protease inhibitors (76). Structural analysis of SFTI-1 shows a well-defined double β -hairpin loop linked by two short antiparallel β -strands (Fig. 4B) (32, 75, 77). SFTI-1 is a potent trypsin inhibitor that belongs to the Bowman-Birk protease inhibitor (BBI) family. The backbone-cyclized SFTI-1 is the smallest and the most potent protease member of the family with a K_i against trypsin in the low nM range (75, 76).

The relatively rigid backbone of SFTI-1 makes its protease-binding loop well-defined, which can serve as a general scaffold for serine protease inhibitors. For example, introduction of several mutations within this loop has produced SFTI-based analogs able to inhibit a variety of serine proteases (78–82). The structural features of SFTI-1 characterized by the presence of a backbone cyclization combined with an internal disulfide bond and an extensive hydrogen binding network make it exceptional stable to thermal or enzymatic degradation (81, 83). SFTI-1 has been also shown to be non-toxic and be able to cross cellular membranes (22). Moreover, as shown with other backbone cyclized disulphide-rich scaffolds, the molecular framework provided by SFTI-1 can also be readily re-engineered by grafting foreign biological active peptide sequences into one the loops producing SFTI-analogs with novel biological activities (84–87).

SFTI-1 has been found to be a potent inhibitor of matriptase ($K_i \approx 1$ nM) (88). Matriptase is a type II transmembrane serine protease found in most cancer cells, where it has been shown that can activate key pro-metastatic substrates to trigger cell migration, cancer invasion, and metastasis; therefore providing a target of therapeutic intervention (89). Amino acid scanning of the position P2' residue (Ile) in SFTI-1 has been also accomplished to produce a potent mesotrypsin inhibitor (90). Replacement of this residue by aromatic residues (Tyr, Phe, Trp or the non-canonical amino acid 4,4'-biphenyl-L-alanine (Bip)) yielded an SFTI-I analog that maintained a similar structure to SFTI-1 and showed marked improvements in activity against mesotrypsin (exceeding 100-fold) (90).

SFTI-1 has also been re-engineered to produce potent human kallikrein-related peptidase 4 (KLK4) inhibitor (82). Human KLK4 is a potential target for prostate cancer treatment because of its proteolytic ability to activate many tumorigenic and metastatic pathways including the protease activated receptors (PARs) (91, 92). In this work, SFTI-1 was modified by introducing several mutations (K5R, T4Q and R2F), identified by using a

combination of molecular modeling and sparse matrix substrate screening, to produce the analog SFTI-FCQR that was potent and selective KLK4 inhibitor ($K_i \approx 3$ nM) (82). Further optimization of the KLK4 inhibitor SFTI-FCQR by the same authors using molecular dynamic algorithms produced a significantly improved inhibitor (SFTI-FCQR Asn14) with a K_i value of ≈ 64 pM (93). The same authors have also employed a similar approach to improve the weaker activity of SFTI-I against cathepsin G ($K_i \approx 570$ nM) through optimization of the binding loop to generate molecules with higher potency ($K_i = 1.6$ nM) and higher selectivity (over 360-fold) for other related proteases (94).

Development of SFTI-based inhibitors of kallikrein-related peptidases 7 (KLK7) and 5 (KLK5) was also recently accomplished by grafting of the reactive-center loop (RCL) of several serpins reported to inhibit KLK5 and KLK7 (95). KLK7 and KLK5 are expressed in human skin, and their dysregulation is associated with skin diseases such as Netherton syndrome, atopic dermatitis, and psoriasis (96); and different types of human cancer (97). The best SFTI-derived inhibitors against KLK7 and KLK5 provide K_i values ranging from 0.6 to 0.9 μ M values.

The SFTI-1 scaffold has been recently used in combination with phage display techniques to screen and select a novel SFTI-based ITG $\alpha_v\beta_6$ -binding peptide (SFITGv6) (98). The ITG $\alpha_v\beta_6$ receptor is highly expressed on head and neck squamous cell carcinoma (HNSCC) and is the target of the antiangiogenic RGD-peptide cilengitide (99). In this work, a linearized version of SFITGv6 peptide labelled with the radiotracer ^{177}Lu -DOTA was employed as bioimaging agent for ITG $\alpha_v\beta_6$ -positive carcinomas (98, 100). SFTI-1 has also been employed to graft the binding loop of micro-protein Min-23 selected to bind angiogenesis marker delta-like ligand4 (Dll4) by using a phage-display approach (101). The micro-protein Min-23 is a two disulfide-bridge stabilized scaffold, which was rationally designed by miniaturization of its parent knottin *Ecballium elaterium* trypsin inhibitor II (EETI-II) (102). In this work, the resulting grafter SFTI-derived peptide was able to preserve the Dll4-binding specificity and the tumor-targeting capability of the original micro-protein (101). A similar grafting approach using anti-angiogenic peptides was also able to yield potent anti-angiogenic SFTI-derived peptides (103). The grafted peptide SFTI-PEDF displayed an almost similar degree of activity as the linear grafted peptide with a better proteolytic stability profile. SFTI-1 has also been transformed into a furin inhibitor by replacing the trypsin-binding loop of SFTI-1 by a natural furin substrate (104). The resulting grafted SFTI-based peptide was further optimized by using computer-based structural modelling to generate a sub-nanomolar furin inhibitor ($K_i \approx 0.5$ nM) that showed very good selectivity over trypsin ($>10,000$) and matriptase ($>1,000$) (104).

The SFTI-1 molecular framework was recently employed to produce a cyclic peptide for for reducing inflammation in models of inflammatory bowel diseases (IBDs) (105). In this work, the authors grafted a small bioactive peptide from the annexin A1 protein into trypsin binding loop of SFTI-1. The resulting SFTI-based peptide (cyc-MC12) maintained the overall fold of the naturally occurring cyclic peptide as more effective at reducing inflammation in a mouse model of acute colitis than the bioactive peptide alone showing enhanced *ex vivo* stability in human serum.

The SFTI-1 scaffold was also recently employed for the production of a novel subtype of selective melanocortin receptor (MCR) agonists (106). This was accomplished by grafting the $\alpha/\beta/\gamma$ -melanocyte stimulating hormone (MSH)-derived HFRW tetrapeptide into the different loops of SFTI-1 in combination with systematic *N*-methylation of the grafted pharmacophore. One of the double *N*-methylated SFTI-derived peptides was able to show low nM activity for human MC1R being about 100 times more selective for this receptor than for MC3R. The nuclear magnetic resonance structural analysis of this grafted peptide revealed the key role of peptide bond *N*-methylation in shaping the conformation of the grafted peptide pharmacophore. This work highlights the potential of cyclic peptide scaffolds for epitope grafting in combination with *N*-methylation to introduce receptor subtype selectivity in the context of peptide-based drug discovery (106).

An interesting application of bioactive grafted SFTI-based peptides was recently reported as a scavenger for anticitrullinated protein/peptide autoantibodies (ACPA) for potential diagnostic and/or treatment of rheumatoid arthritis (107). In this interesting work, the SFTI-1 scaffold was engineered to display a citrulline-containing ACPA binding epitope identified from the α -chain of human fibrinogen. The resulting cyclic peptide scavenger showed high apparent affinity and subtype-specific binding for ACPA, as well as superior serum stability.

All these examples show the great potential of SFTI-1 as molecular framework for grafting or engineering new activities. Its small size and high resistance to proteolytic degradation are making it increasingly recognized as an excellent template for engineering studies as it does not require structural optimisation and the inhibitor's inherent activity can be re-directed to other serine proteases by substituting residues that form major binding contacts.

Concluding remarks

It is fair to say that the use of highly constrained disulphide-rich backbone cyclized polypeptides is becoming to gain acceptance as molecular scaffolds for the potential design of novel peptide-based therapeutics and diagnostic tools. This is in part to their unique properties which include their small size that allows chemical synthesis, the ability to be expressed using standard expression systems, high resistance to chemical, physical and biological degradation and in some case the ability to cross cellular membranes to target intracellular PPIs.

Of the three types of scaffolds reviewed here, the cyclotide scaffold without question is one of the more exciting. The cyclotide unique knotted arrangement of three disulfide bonds and exceptional tolerance sequence variation in all their hypervariable loops provides an exceptional molecular platform to design novel cyclotides with new biological activities by rational design using molecular grafting techniques or by employing molecular evolution techniques making use of the multiple loops found on the cyclotide scaffold. Cyclotides have also been shown to cross mammalian cellular membranes to target protein-protein interactions *in vitro* but also and more importantly in animal models. This highlights the high stability of the Cys-knot to be degraded/oxidized under complex biological conditions.

As mentioned earlier, the relative small size of these scaffolds facilitates their chemical synthesis allowing the introduction of chemical modifications such as non-natural amino acids and PEGylation to improve their pharmacological properties.

None of the scaffolds reviewed here have reached human clinical trials yet, however, the results obtained with several bioactive compounds in animal models may hint that this could occur in a not too distant future. One of the main challenges that affect this type of constrained polypeptides is their oral bioavailability if they want to be competitive with small-molecule therapeutics. Although we have seen that some cyclotides have been shown to be orally active, there is still little information about their oral bioavailability. It is anticipated, however, that more studies on the biopharmaceutical properties of these exciting new peptide-based molecular scaffolds will be available very soon.

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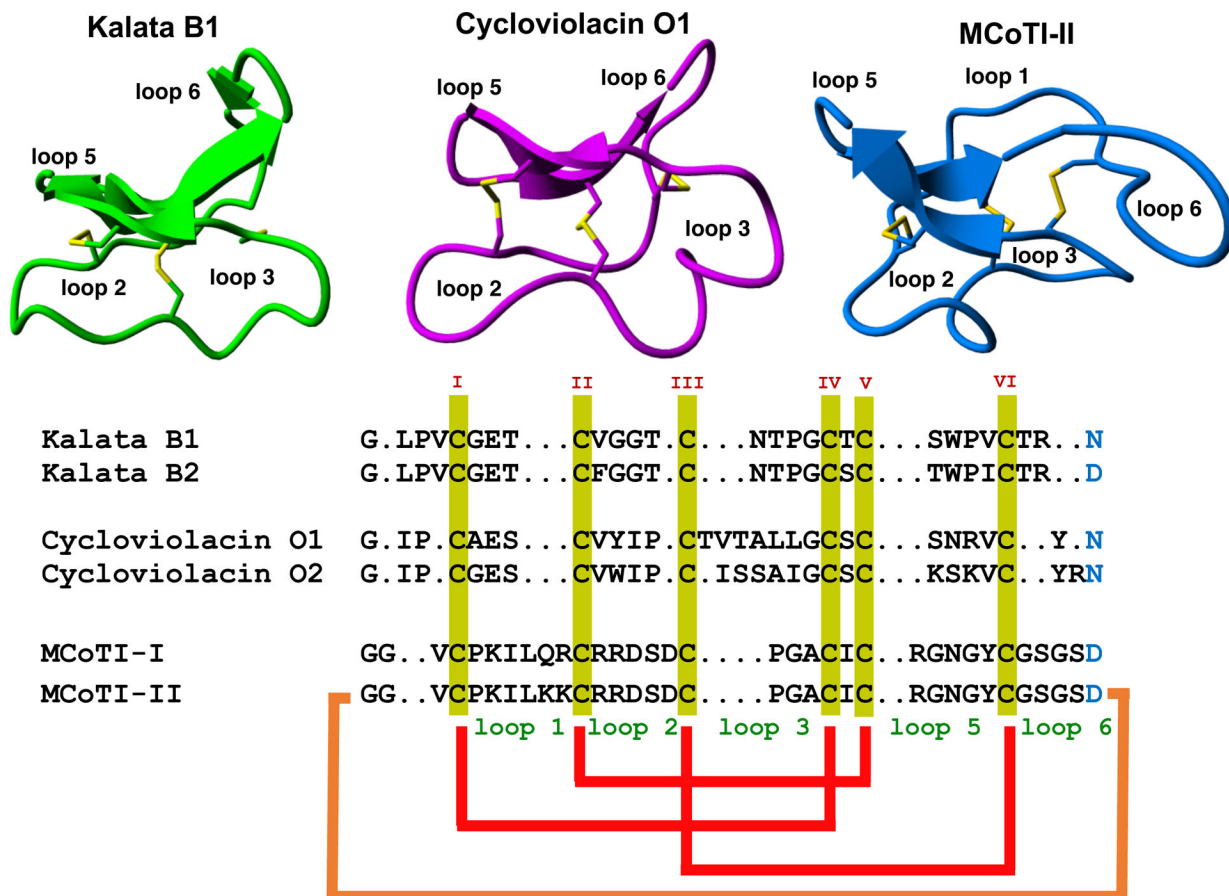


Figure 1.

Sequence alignment and structures of different cyclotides belonging to the Möbius (kalata B1, pdb: 1NB1), bracelet (cycloviolacin O1, pdb: 1NBJ) and trypsin inhibitor (MCoTI-II, pdb: 1IB9) subfamilies. Disulfide connectivities and backbone-cyclization are shown in red and orange, respectively. The six Cys residues are labeled with roman numerals whereas loops connecting the different Cys residues are designated with arabic numerals. Conserved Cys and Asp/Asn (required for backbone cyclization in nature) residues are marked in yellow and light blue, respectively. Molecular graphics were created using Yasara (www.yasara.org).

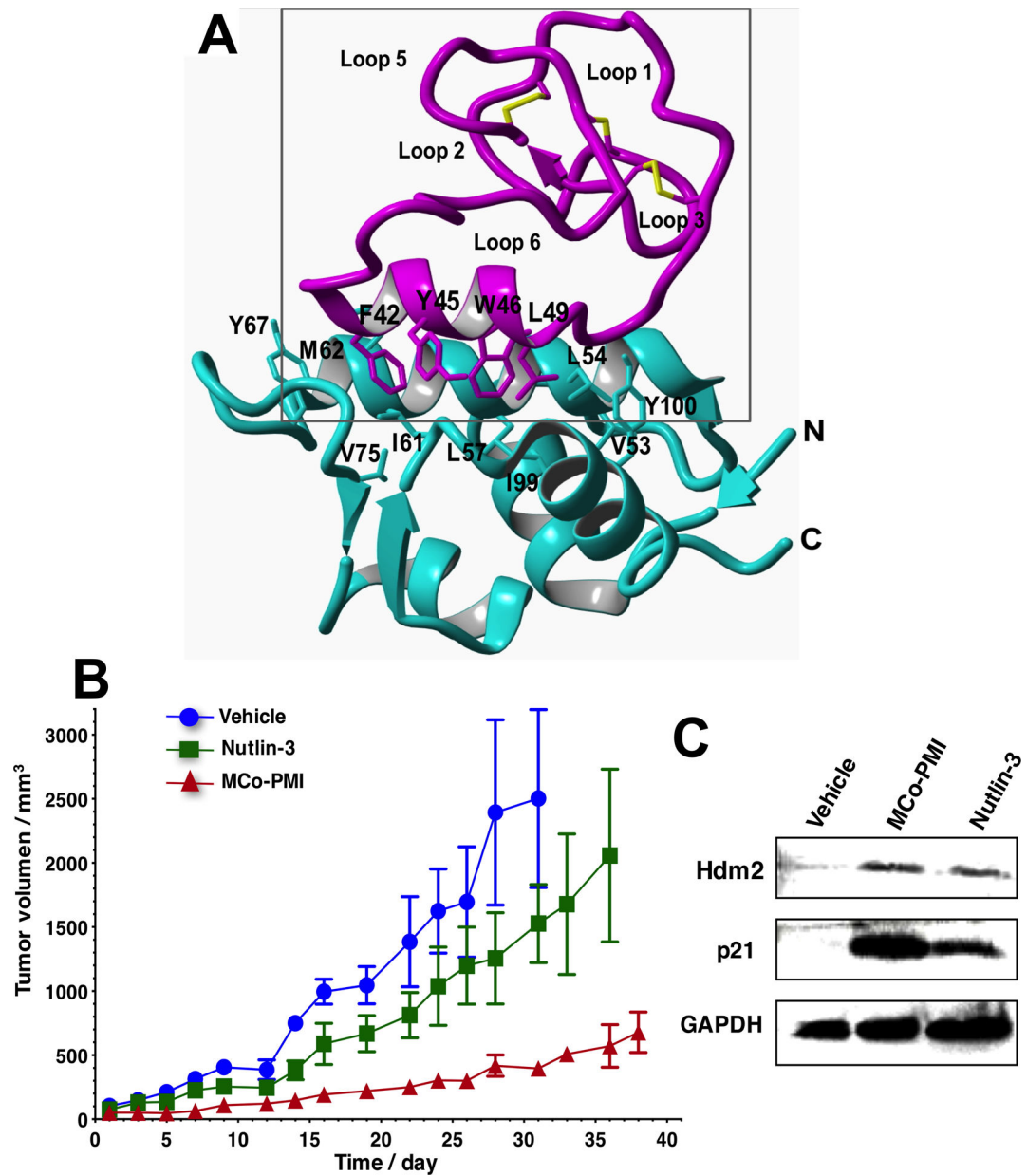


Figure 2. Structure and *in vivo* activity of the first cyclotide designed to antagonize an intracellular protein-protein interaction *in vivo* (27). **A.** Solution structure of the engineered cyclotide MCo-PMI (magenta) and its intracellular molecular target, the p53 binding domain of oncogene Hdm2 (blue) (pdb: 2M86). The cyclotide binds with low nM affinity to both the p53-binding domains of Hdm2 and HdmX. **B.** Cyclotide MCo-PMI activates the p53 tumor suppressor pathway and blocks tumor growth in a human colorectal carcinoma xenograft mouse model. **C.** Tumors samples were subjected to SDS-PAGE and analyzed by western blotting for p53, Hdm2 and p21, indicating activation of p53 on tumor tissue.

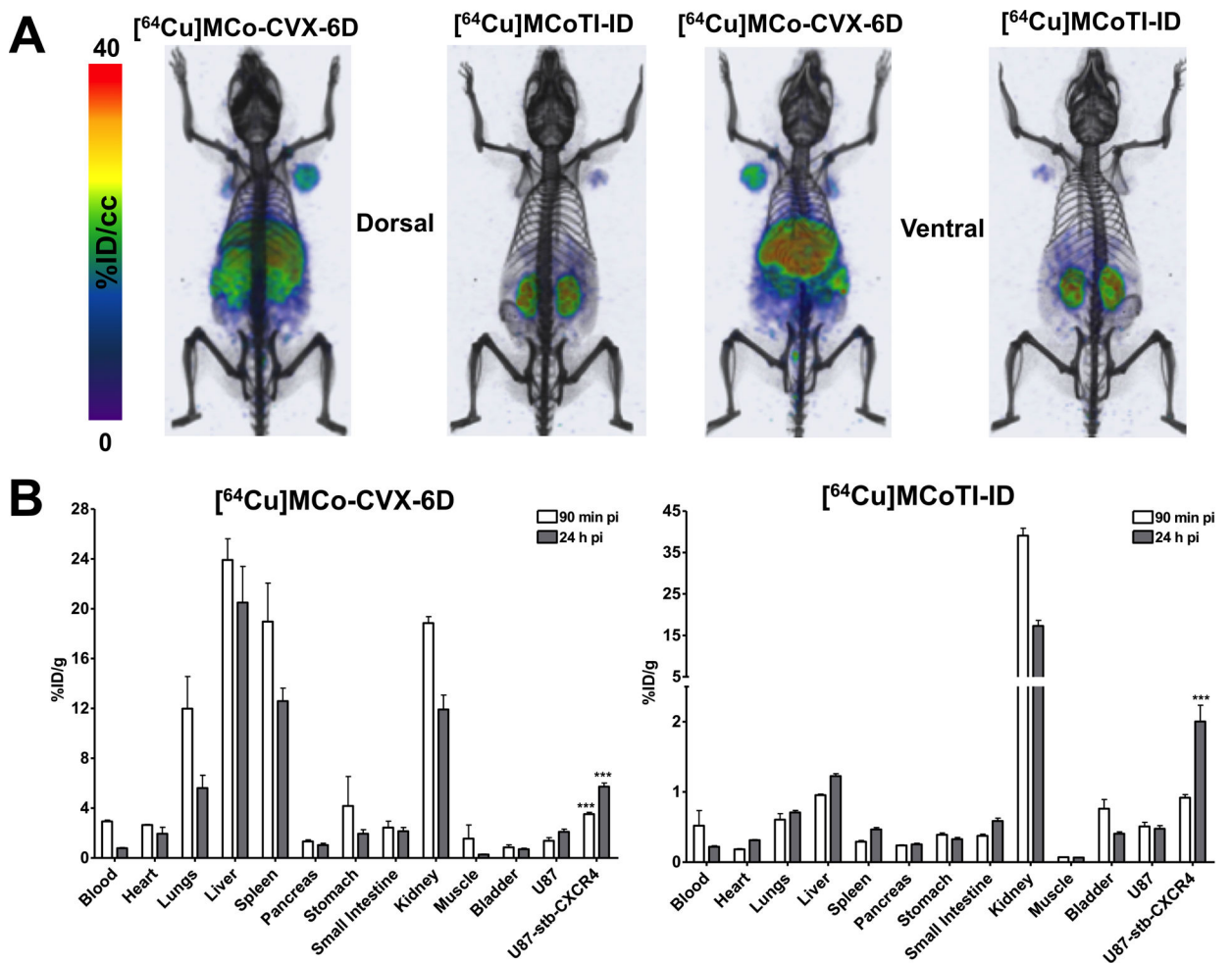


Figure 3.

Use of a CXCR4-targeting cyclotide as a bioimaging tool for detecting CXCR4-overexpressing tumor cells in animal models (24). Distribution of [⁶⁴Cu]MCo-CVX-6D (CXCR4-targeting cyclotide) and [⁶⁴Cu]MCoTI-ID (a DOTA-labeled variant of native trypsin inhibitor cyclotide MCoTI-I) in NOD/SCID mice bearing U87 and U87-stb-CXCR4 tumors with PET-CT. **B.** *Ex vivo* evaluation of [⁶⁴Cu]MCo-CVX-6D and [⁶⁴Cu]MCoTI-ID distribution and specificity in NOD/SCID mice bearing U87 and U87-stb-CXCR4 tumors. *Ex vivo* biodistribution analysis was performed at 90 min and 24 h after post tracers injection.

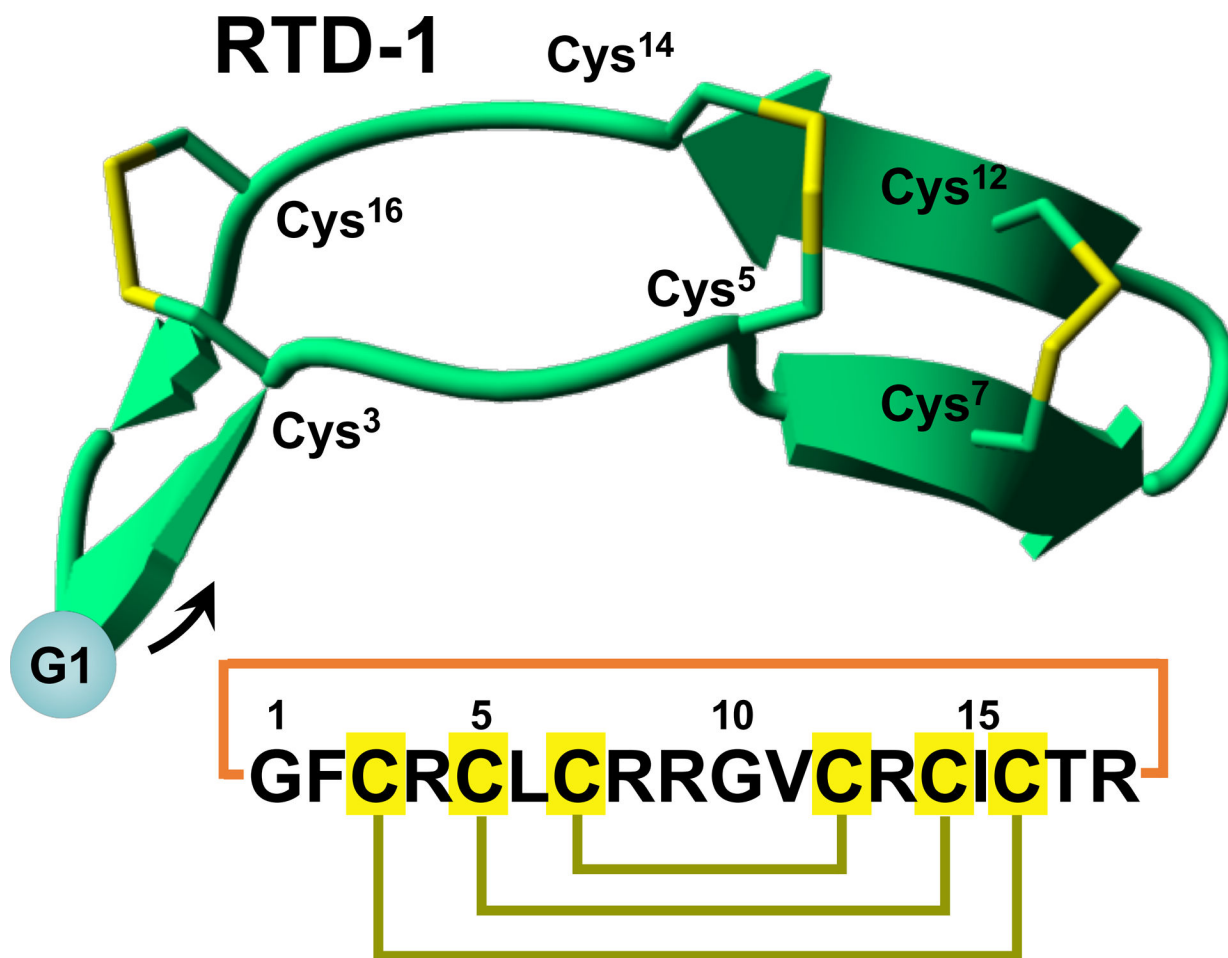
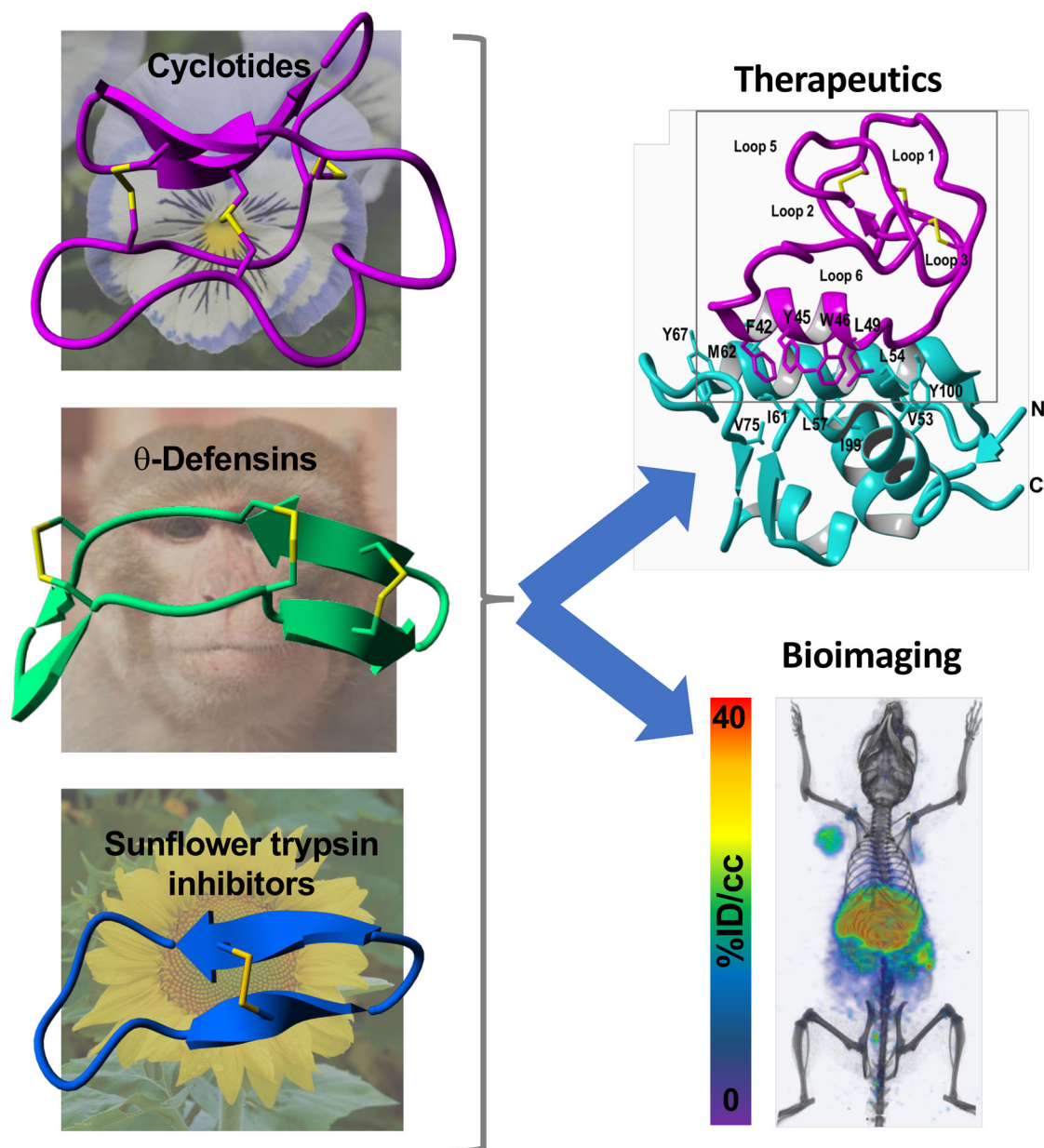


Figure 4. Sequences and structures of naturally-occurring disulfide-rich backbone cyclized peptides θ -defensin (RTD-1) (A) and sunflower trypsin inhibitor 1 (SFTI-1) (B). The backbone cyclized peptide (connecting bond shown in blue) is stabilized by the three disulfide bonds in a ladder formation (disulfide bonds shown in yellow). Molecular graphics were created using Yasara (www.yasara.org).

**Scheme 1.**

Schematic representation of the structural complexity of the naturally occurring disulfide-rich backbone cyclized peptides that are commonly used as tools for the development of novel bioimaging agents (left, depicting a cyclotide able to visualize CXCR4-expressing tumors *in vivo*) and therapeutic leads (right, depicting a cyclotide able to activate the p53 pathway in cancer cells *in vivo*). These include θ -defensins (RTD-1), cyclotides (cycloviolacin O2) and sunflower peptide trypsin inhibitors (SFTI-1).

Table 1.

Summary of work published in engineered cyclotides with novel biological activities leading to therapeutic and bioimaging applications.

Cyclotide	Biological activity	Loop Modified	Application	Ref.
Möbius subfamily				
Kalata B1	VEGF-A antagonist 2	2, 3, 5 & 6	Anti-angiogenic, potential anti-cancer activity	(41)
Kalata B1	Dengue NS2B-NS3 Protease inhibitor	2 & 5	Anti-viral for Dengue virus infections	(108)
Kalata B1	Bradykinin B1 receptor antagonist	6	Chronic and inflammatory pain	(45)
Kalata B1	Melanocortin 4 receptor Agonist	6	Obesity	(44)
Kalata B1	Neuropilin-1/2 antagonist	5 & 6	Inhibition of endothelial cell migration and angiogenesis	(109)
Kalata B1	Immunomodulator	5 & 6	Protecting against multiple sclerosis	(110)
Kalata B1	Immunomodulator	4	Protecting against multiple Sclerosis	(111)
Trypsin inhibitor subfamily				
MCoTI-I	CXCR4 antagonist	6	Anti-metastatic and anti-HIV PET-CT imaging	(24–26)
MCoTI-I	p53-Hdm2/HdmX Antagonist	6	Anti-tumor by activation of p53 pathway	(27)
MCoTI-II	FMDV 3C protease Inhibitor	1	Antiviral for foot-and-mouth disease	(43)
MCoTI-II	β -Tryptase inhibitor	3, 5 & 6	Inflammation disorders	(49)
MCoTI-II	β -Tryptase inhibitor Human elastase inhibitor	1	Inflammation disorders	(42)
MCoTI-II	CTLA-4 antagonist	1,3 & 6	Immunotherapy for cancer	(54)
MCoTI-II	Tryptase inhibitor	1	Anticancer	(81)
MCoTI-II	VEGF receptor agonist	6	Wound healing and cardiovascular damage	(87)
MCoTI-I	α -Synuclein-induced cytotoxicity inhibitor	6	Parkinson's disease Validate phenotypic screening of genetically-encoded cyclotide libraries	(40)
MCoTI-II	BCR-Abl kinase Inhibitor	1 & 6	Chronic myeloid leukemia Attempt to graft both a cell penetrating peptide and kinase inhibitor	(112)
MCoTI-I	MAS1 receptor agonist	6	Lung cancer and myocardial infarction	(113)
MCoTI-II	SET antagonist	6	Potential anticancer	(50)
MCoTI-II	FXIIa and FXa inhibitors	1 & 6	Antithrombotic and cardiovascular disease	(114)
MCoTI-II	Thrombospondin-1 (TSP-1) agonist	6	Microvascular endothelial cell migration inhibition Anti-angiogenesis	(85)
MCoTI-II	Antiangiogenic	5 & 6	Anti-cancer	(103)