The Therapeutic Potential of Cystine-Knot Microproteins

Cystine-knot microproteins are a class of very small, highly stable proteins that can be administered orally and have considerable therapeutic potential as potent, selective modulators of biomolecular interactions.

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Owing to their inherent extraordinary thermal and biological stability and their small size, cystine-knot microproteins provide attractive scaffolds for the development of peptide-based pharmaceuticals. They are readily accessible through recombinant bacterial production or solid phase chemical synthesis. Highly potent and selective microproteins with predefined binding characteristics have been obtained by rational protein design as well as by combinatorial library screening. Moreover, oral administration of microproteins seems to be feasible. With the first natural microprotein already successfully marketed as an analgesic it can be expected that tailor-made therapeutic candidates will follow over the next years.

DISTRIBUTION IN NATURE

Cystine-knot microproteins are broadly distributed in nature and display a plethora of therapeutically useful biological activities. They are very small (typically around 30 amino acids in length) and have in common an extremely stable tertiary fold, held together by a characteristic pattern of three disulfide bonds – the so-called cystine knot (see Figure 1). In contrast to larger proteins, their conformation is thus mainly stabilised by intramolecular covalent linkage rather than by noncovalent interactions, thereby explaining their extraordinary stability. They can be boiled, incubated at 65°C for weeks and even placed in 1 N HCl or 1 N NaOH, without any loss of structural or functional integrity. Moreover, many microproteins are resistant to proteolytic attack. It is this combination of high chemical/biological stability and high potency/selectivity that makes them promising candidates for many applications including drug development (1).

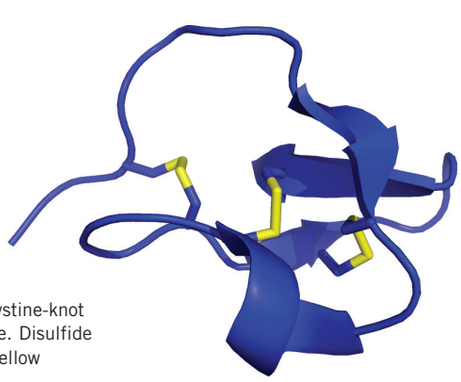

Figure 1: Schematic representation of a cystine-knot microprotein structure. Disulfide bonds are shown in yellow

Cystine-knot microproteins are found in many diverse natural sources, such asporifera, arthropoda, mollusca, vertebrata, fungi and plantae. Many microproteins that target voltage-gated ion channels have been isolated from marine cone snails. The most prominent example is ω-conotoxin MVIIa, a potent calcium channel blocker derived from the mollusc *Conus magus*. First clinical trials of ω-conotoxin MVIIA (ziconotide) for the treatment of brain ischaemia were conducted in 1993. In March 2000, ziconotide (Elan Pharmaceuticals) completed Phase III clinical trials as an analgesic for the treatment of malignant and non-malignant neuropathic pain, and in 2004 it was approved by the US FDA as Prialt® for the treatment of

severe chronic pain (2). Several other conotoxins are in Phase I/IIa stage clinical trials, and more are expected to come through for the treatment of neurological disorders.

Another large group of cystine-knot microproteins are found in squash and act as serine protease inhibitors. Their inhibiting function is located within a loop with canonical conformation between the first and the second cystine residue of the microprotein sequence. Such loops inhibit the proteolytic activity of serine proteases by protruding into their active sites, often even with picomolar inhibition constants (3). Moreover, many plant species contain cyclic microproteins – cyclotides – that share the interlocking arrangement of the three disulfide bonds and in addition have a head-to-tail cyclised peptide backbone; this architecture results in a structural framework that is even more constrained than in the open chain analogues. Cyclotides are well-known for their diverse spectrum of biological activities. Reports of African natives using a concoction of the plant *Oldenlandia affinis* as an oral oxytocic led to discovery of the cyclotide Kalata B1, a pharmacologically active compound that mediates an uterotonic effect. Other cyclotides with insecticidal, haemolytic, cytotoxic and anti-HIV activity have also been described (1).

DESIGNING NEW DRUG CANDIDATES

Several protein engineering efforts have been made to construct microproteins with predefined specificities to target proteins of therapeutic relevance. It is well-known that microproteins tolerate changes – such as substitution of individual amino acids, replacement of whole loop sequences or insertion of additional amino acids – without any loss of structural integrity. In one of the first examples of microprotein functionalisation, peptides with known activity against the $\alpha_{IIb}\beta_3$ receptor of platelets were grafted into a microprotein scaffold by replacement of a natural loop sequence. The engineered microproteins were 100-fold more potent in inhibiting fibrinogen binding, $\alpha_{IIb}\beta_3$ activation and platelet aggregation compared with the linear RGD and KGD peptides (4).

Of potential therapeutic relevance is the development of microproteins that inhibit human mast cell tryptase. The terameric human β-II tryptase is believed to play an important role in allergic asthma and almost exclusively resides within mast cells. From virtual docking experiments of the squash-derived microprotein McoTI-II to the tryptase tetramer, it was concluded that four microprotein molecules might fit into the central cavity of the tryptase tetramer simultaneously. However, none of the three natural trypsin inhibitors isolated from the squash plant *Momordica cochinchinensis* – two being cyclic and one being linear – displayed inhibitory activity against the human enzyme. An open chain variant of the natural cyclic microprotein McoTI-II lacking the loop sequence connecting the N- and C-terminus, displayed tryptase inhibition with a K_i of 100nM. Further affinity improvement by structure-based design resulted in a cyclic microprotein with a 1nM inhibition constant; this is the most potent proteinaceous inhibitor of human mast cell tryptase known to date and is able to block all four active sites of the tryptase tetramer simultaneously (5).

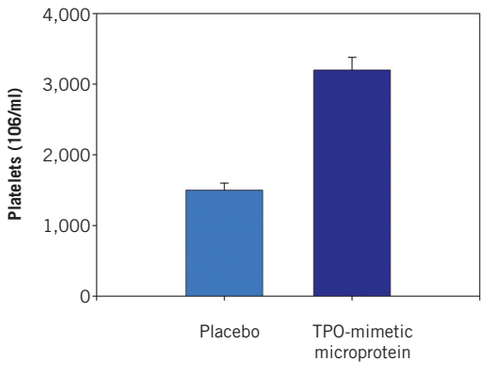

Figure 2: Thrombogenic activity of a TPO-mimetic microprotein dimer in mice

Most natural microproteins act as inhibitors. We recently succeeded in functionalising a microprotein to act as an activator of thrombopoiesis in a thrombopoietin-like manner, with a mean effective concentration of 100pM. This was achieved by introducing a 14-mer peptide into a squash and a human microprotein scaffold. These variants were shown to block antagonistically thrombopoietin-mediated receptor activation. It is important to know that dimerisation of the thrombopoietin receptor is required for receptor activation. Covalent chemical linkage of the antagonistic microproteins yielded agonistic dimers, being almost as potent as natural thrombopoietin with respect to stimulation of megakaryocyte colony formation from human bone marrow mononuclear cells (6). Moreover, in animal studies they elicited a doubling of platelet counts in mice when administered subcutaneously (see Figure 2). This suggests that designed dimeric cystine-knot microproteins may have considerable potential for the future development of small and stable receptor agonists.

There is a growing number of examples of the successful screening of microprotein-based random peptide libraries. For example, a peptide library based on the microprotein EETI-II was generated where six residues of the first loop were randomised. The constrained library was screened against the natural target of wild-type EETI-II, bovine trypsin, and allowed for the identification of a minimal consensus trypsin-binding motif (7). In another example an amino terminal-shortened 23-residue version of EETI-II, lacking the first disulfide bond but retaining the cystine, stabilised β-sheet motif was used as a scaffold for the conformationally constrained insertion of ten randomised amino acids on a β-turn. The resulting phage display library of more than 10^8 variants was successfully

screened against – amongst others – malarial antigen AMA-1, the mitochondrial membrane protein Tom70 and the HIV viral protein Nef (8). These examples indicate that screening of constrained random peptide libraries that are embedded into a microprotein scaffold may in the near future provide a range of novel compounds for pharmaceutical applications.

POTENTIAL ROUTES TO ORAL ADMINISTRATION

Cystine-knot microproteins exhibit several properties that make them very interesting as scaffolds for the oral delivery of peptide-based drugs. Apart from the absorption barrier, the most important barrier for orally administered peptides and proteins is enzymatic degradation, especially by proteolytic enzymes residing in the brush border membrane of the intestinal mucosa. Several microproteins have been identified that show low proteolytic susceptibility and longterm stability at elevated temperatures and under extreme pH conditions. Furthermore, some cyclic microproteins have been found to be completely resistant to degradation by soluble gastrointestinal proteases such as trypsin, pepsin, chymotrypsin or elastase. Open chain microproteins show some susceptibility but this is nevertheless low compared with other proteins or peptides. Moreover, it has been demonstrated that preferred cleavage sites in the microprotein backbone can be replaced by other residues leading to further stabilisation against specific proteolytic attack.

Most importantly, cystine-knot microproteins have been found to be resistant to proteolytic enzymes in the brush border membrane, which readily cleave most natural proteins and peptides. In addition, for two microproteins, it was shown recently that permeation through excised rat intestinal mucosa was more efficient than for the reference peptide drugs insulin and bacitracin, a 12-mer peptide (see Figure 3) used as an antibiotic in animal health care and administered orally (9,10). More detailed animal studies with microprotein-based drug candidates are required to validate these promising *in vitro* findings and develop the potential of cystine-knot microproteins as a novel class of stable, potent and – possibly – orally available biomolecules.

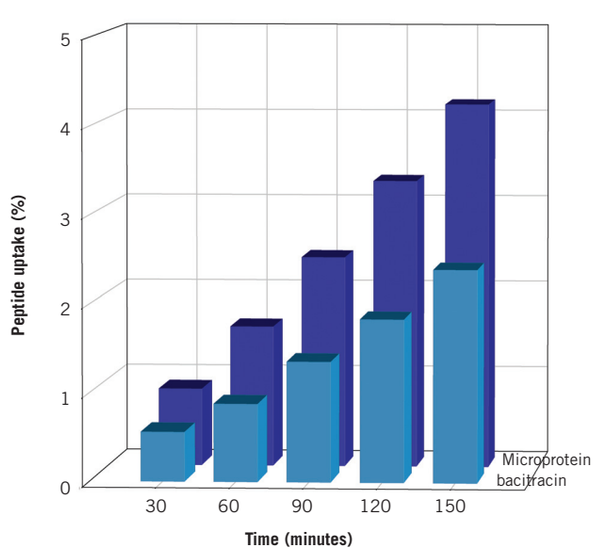

Figure 3: Trans-epithelial uptake of a microprotein by rat intestinal mucosa

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Notes

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References

1. **Craik D *et al*, The chemistry and biology of cyclotides, *Curr Opin Drug Discov Devel* 10, pp176-84, 2007**
2. **Klotz U *et al*, Ziconotide – a novel neuron-specific calcium channel blocker for the intrathecal treatment of severe chronic pain – a short review, *Int J Clin Pharmacol Ther* 44, pp478-483, 2006**
3. **Chiche L *et al*, Squash inhibitors: from structural motifs to macrocyclic knottins, *Curr Protein Pept Sci* 5, pp341-349, 2004**
4. **Reiss S *et al*, Inhibition of platelet aggregation by grafting RGD and KGD sequences on the structural scaffold of small disulfide-rich proteins, *Platelets* 17, pp153-157, 2006**
5. **Avrutina O *et al*, Head-to-tail cyclized cystine-knot peptides by a combined recombinant and chemical route of synthesis, *Chembiochem* 9, pp33-37, 2008**
6. **Krause S *et al*, Grafting of thrombopoietin-mimetic peptides into cystine knot miniproteins yields high-affinity thrombopoietin antagonists and agonists, *Febs* J 274, pp86-95, 2007**
7. **Baggio R *et al*, Identification of epitope-like consensus motifs using mRNA display, *J Mol Recognit* 15, pp126-134, 2002**
8. **Souriau C *et al*, New binding specificities derived from Min-23, a small cystine-stabilized peptidic scaffold, *Biochemistry* 44, pp7,143-7,155, 2005**
9. **Werle M *et al*, The potential of cystine-knot microproteins as novel pharmacophoric scaffolds in oral peptide drug delivery *J Drug Target* 14, pp137-146, 2006**
10. **Werle M *et al*, Evaluation and improvement of the properties of the novel cystine-knot microprotein McoEeTI for oral administration, *Int J Pharm* 332, pp72-79, 2007**