The Nature of Peptidomimetics: Synthetic Peptide Macrocycles as Potential Therapeutics and Artificial Peptide Helices

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Introduction

Macromolecular structure plays a large role in peptide cellular function. Cyclic peptides comprise a unique class of molecules with much potential in novel drug development. By restricting bond rotation, cyclization can constrain a peptide into a favorable conformation, limiting the degrees of freedom while promoting a larger surface area of interaction with a biological target of interest. Reports on macrocyclic peptide drugs extol their superior characteristics and benefits towards drug oral bioavailability, which includes enhanced intracellular stability as their conformation is less accessible to proteases [1]. Throughout the literature, studies have confirmed that macrocyclization improves drug proteolytic stability, pharmacokinetics and decreased side effects [2]. Given these attributes, further development of cyclic peptide-based therapies is necessary.

Endocrine hormone vasopressin is a small, macrocyclic peptide that controls water balance and

blood pressure by acting on the kidneys [3]. Low concentrations of vasopressin are inherent in the disease Neurogenic Diabetes Insipidus (DI), characterized by extreme dehydration and electrolyte imbalance. Current therapies are limited by the necessity of injection and the structural weaknesses in the backbone of most common drugs. We describe two specific vasopressin designs and one that mimics structurally-related hormone oxytocin (Figure 1a). Our peptides use head-to-tail amide bond cyclization, removing the cysteine-cysteine disulfide connection that could render the peptide linear via exchange in the cell's reducing environment (Figure 1a) [3]. The overall arrangement of amino acids in each design is similar to the parent compounds with the exception of the addition of non-natural diaminopropionic acid (Figure 1a), to emulate the N-terminal region on the hormones, and a glycine

Fig. 1. a) Chemical structures of vasopressin, oxytocin and the vasopressin and oxytocin mimics [3]; b) Chemical structures of literature N- and Q-peptides [6] and the HTN- and Q-pep mimics; c) Sequences for the α -peptides (top) and β -peptides (bottom) described herein with helical wheels and helical net diagrams depicting possible helical arrangement [7].

residue to provide flexibility reminiscent of the disulfide region. The additional tyrosine in VP mimic2 (Figure 1a) was included because studies in the literature show short, linear peptides with three aromatics improved affinity for vasopressin's targets. Each of our designs is smaller, more compact, and retains the residues of utmost importance while removing the 'tail' of three amino acids external to the ring not crucial for binding interactions [3]. On the whole, the mimics remain similar to the parent compounds in structure and design with deliberate differences for improving stability and efficacy. In our study we examined the stability of each compound when treated with a panel of proteases (Figure 2a).

In current research, we employ a similar design strategy developing potential thrombosis inhibitors. Rupturing of atherosclerotic plaques exposes collagen fibrils in arterial vessel walls which creates high shear pathological blood flow [4]. Von Willebrand Factor (vWF) is a multimeric protein essential to the recruitment and adhesion of platelets at these sites of vascular injury [4]. It has distinct domains that bind collagen tightly and bridge platelets through interactions with glycoproteins on the platelet surface [5]. Once an initial layer of platelets are attached, they can accumulate leading to thrombus growth. vWF is intrinsic to both initial platelet tethering and encouraging the growth of the platelet plug. Given the gravity of thrombus formation, and the severe side effects of most anti-platelet medications, there is a significant need for anti-thrombosis pharmaceutical research. Combining the benefits of macrocyclic drugs with the importance of inhibiting vWF in blood clot formation, we designed novel cyclic peptides that target the interface between vWF and collagen, in hopes to prevent nucleation of blood clot formation by inhibiting this initial interaction. There currently exists a literature precedent of cyclic peptides, comprising a region of vWF, found through screening a phage display library that inhibit vWF binding collagen [6]. These compounds displayed IC_{50} values in the moderate mid-micromolar range and were cyclized naturally through disulfide bonds prone to the linearization and enzyme cleavage mentioned above [6]. Starting with these compounds, we developed potential first generation peptide-based therapeutics using our head-to-tail synthesis (Figure 1b). Similar to the vasopressin/oxytocin mimics above, our anti-thrombosis peptides contain a glycine in place of the disulfide bond. Two designs (HTNPepDap and HTQPepDap) utilize diaminopropionic acid to emulate the *N*-terminal region of the parent compounds, however remove the valine residue from the ring, to promote aqueous solubility. Both HTNPepT and HTQPepT replace the valine with a threonine, also to increase solubility. Our ongoing studies include stability assays against the same

Fig. 2. a) Results from protease stability assay from vasopressin and oxytocin study; b) Results from protease stability assay thus far on the anti-thrombosis peptides; c) Results from validation of ELISA, vWF binding collagen in a dose dependent manner to $K_D = 0.0003 \pm 5.4 \times 10^{-5}$ mg/mL.

protease panel (Figure 2b) as well as competition Enzyme-Linked Immunosorbent Assays to determine whether the cyclic peptides can inhibit vWF binding of collagen.

Frequently, the relationships between proteins are implicated in the disease state and can be traced to interactions between secondary structures of the entities involved. The α -helix comprises the major secondary structure found in natural proteins, playing a functional role in a vast majority of proteins found in the cell [7]. Many

helical interactions have been defined and their mis-regulation implicated in the disease state. In the continuing pursuit of novel peptide-based therapeutics, a better understanding of what dictates α -helical structure is necessary. Additionally, the field of peptidomimetics is rife with examples of non-natural folding oligomers with biological properties. β -peptides, which are oligomers of β -amino acids bearing an extra carbon along the amino acid backbone, can fold into a variety of helices. The 14-helix is most prevalent for peptides comprised of amino acids with substituents in the R3 position, and resembles the native α -helix [7]. There are β -peptides that demonstrate helicity as well as applied biological function, such as inhibiting protein-protein interactions involved in disease. While helical regions in the context of a protein tend to be 10-11 residues, a small subset of two or three amino acids along one side of a helix called “hot spots” contribute most to the binding between proteins [7]. Therefore, an ideal peptidomimetic would be a relatively short peptide of defined structure. To further our understanding of helical control as well as inform the future design of novel short, folded peptides we directly compared the relationship between primary sequence and helicity in 6-8 residue α - and β -peptides using circular dichroism (CD) (Figure 1c). PT2-PT4 are designed to maximize α -helicity, using Aib residues, Leucine zipper or Alanines, respectively [7]. BP1 is designed to maximize 14-helicity, using an established β -peptide model motif, cut down to the smallest possible epitope in 6 residues. PT1 is the “ α -equivalent” of the BP1 design, and BP2-4 are the “ β -equivalent” of the α -peptide designs.

Results and Discussion

The head-to-tail cyclized vasopressin and oxytocin mimics degraded much less and with overall superior stability when compared to vasopressin, oxytocin and a linear control peptide upon treatment with digestive proteases. We used an equation to calculate percent degradation that equates UV absorbance and HPLC peak height to the concentration of peptide in solution. Each solution was treated with the enzyme in the presence of glutathione, a reducing agent often found in the cell which could render the disulfides of the parent compound linear. Pepsin, a stomach enzyme that targets consecutive aromatic residues, degraded the linear control nearly 100% in an hour of treatment, and vasopressin about 80% overall, as expected since all of the compounds contain aromatic residues (Figure 2a) [3]. In comparison, VP mimic2 degraded on average about 5-10%, VP mimic1 about 2.5-4% and OT mimic1 is most stable with only 2-2.5% degradation (Figure 2a) [3]. Alpha-chymotrypsin, an intestinal digestive enzyme which targets aromatic residues, was found to degrade the linear sequence to about 75%, before leveling off, whereas vasopressin and oxytocin were found to degrade to about 50-75%, with vasopressin the more stable of the two parent compounds (Figure 2a) [3]. Each of the hormone mimics demonstrated significantly more stability as compared, with degradation levels at 20% or below [3]. The VP mimics were predictably less stable than the OT mimic, as they contain one or two more aromatics than the OT mimic. Pronase, the most severe test,

Fig. 3. CD spectra of 3_{10} / α -helical PT 2-4; PT3 most helical (28% calculated at 222 nm) and 14-helical BP1 and BP2; BP1 most helical (64% calculated at 214 nm), 100 μ M, 50 μ M and 25 μ M overlaid.

is actually a mixture of protease enzymes with the ability to cleave nearly every amide bond. As per the other assays, the linear sequence most rapidly degraded over the time course, however in this case oxytocin showed a similar complete degradation after about an hour incubation. Vasopressin had an

unprecedented stability against prolonged pronase treatment with over 50% remaining after 2 hours [3]. Uniquely, VP mimic2 actually showed steady degradation with only about 40% of its original quantity present after 2 hours. VP mimic1 and OT mimic1 were surprisingly unreactive, showing only about 1% and 5-10% degradation, respectively. The sequence of these peptides differ by only one amino acid and this astounding result showed that each peptide had extraordinary stability against the most rigorous protease test. Overall, the three head-to-tail cyclized peptide mimics showed exceptional stability and would make a good prototype for a first generation pharmaceutical.

Continuing work with cyclic peptides as first generation antithrombosis drug leads involved the HTN and Q pep series targeting vWF binding to collagen. These peptides illustrated similar levels of stability when treated with the same panel of proteases. Still in progress, the results from each of the cyclic peptides treated against pepsin, alpha-chymotrypsin and pronase has yielded $\leq 30\%$ degradation for each compound (Figure 2b). Ongoing parallel protease studies on the parent N- and Q-peptides bearing disulfide bonds and a linear vWF peptide will be performed for comparison. The ELISA has been validated using serial dilutions of vWF to achieve a dose dependent response curve that specifies the binding of vWF to collagen (Figure 2c). Plates pre-coated with collagen are incubated with vWF followed by an anti-vWF antibody linked to an assayable horse radish peroxidase enzyme. Upon treatment with a substrate the fluorescent product indicates the presence of vWF. Currently, studies are underway to use this method in conjunction with serial dilutions of cyclic peptide as competitor to view the degree by which fluorescence decreases to indicate the inhibitory effect of the cyclic peptide.

Results from our helical study showed that short 6-8 residue α - and β -peptides can be designed with specific sequences to control the type of secondary structure into which they fold. However, when directly comparing α - and β -peptides we found that primary sequences that control helicity in one type of peptide cannot be substituted into peptides of a different type; optimal designs are generally specific to α - or β -peptides for α - or 14-helical arrangements, respectively. We saw no initiation of cross helicity, and confirmed this by CD spectroscopy (Figure 3). Interestingly, PT2 contained some α -helical structure, whereas BP2, the β -equivalent, had some 14-helical structure, showing that there was some minor overlap in sequence-based control. BP1, the most intensely 14-helical β -peptide, showed a remarkably strong signature considering its short scaffold of only 6 amino acids. This peptidomimetic could be of future use in emulating peptides with biological function. PT3 and, to a lesser extent, PT2 and PT4 showed the possibility of some 3_{10} -helical signature, as well, which is thought to be an intermediate in α -helical folding. Overall, the peptides revealed interesting information about helical control and also paved the way for novel short 3_{10} -/ α -helical peptides as well as 14-helical β -peptides that can be used to probe protein-protein interactions involving short "hot spot" regions.

We conclude that the structure, whether cyclic or helical, of peptides and their peptidomimetics is key in terms of controlling how they fold and function. Our designs move in the direction of novel pharmaceutical design.

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