The importance of amide protons in peptide drug development

Anthony Harrington and Yftah Tal-Gan*

Department of Chemistry, University of Nevada, Reno, 1664 North Virginia Street, Reno, Nevada 89557, United States

* To whom correspondence should be addressed: ytalgan@unr.edu

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Background

Peptide-based therapeutics are steadily increasing with over 60 drugs currently prescribed in the United States and over 100 in clinical trials.[1] Contributions from structure-activity relationship studies help identify key residues, conformational constraints, and other features necessary to rationally design therapeutic peptide activators and inhibitors.[3,2] Alanine, D-amino acid and *N*-alkyl amino acid scanning are the most common screenings that identify key residues (alanine scan), each residue's conformational importance (D-amino acid scan) and the importance of each residue's amide proton (*N*-alkyl amino acid scan). *N*-alkyl amino acid scanning identifies residues whose amide protons participate in H-bonding with their target, amide bond positions that are targets for enzymatic degradation and amide protons that stabilize conformations through intra-molecular H-bonding.[3-5] The simplest and most widely used *N*-alkyl modification is *N*-methylation (N-Me), which is commonly introduced to peptide sequences through the incorporation of commercially available *N*-methyl amino acids during solid-phase peptide synthesis. Many naturally occurring peptides containing backbone *N*-methylation have been discovered and their therapeutic potential and properties are being exploited to develop new drug leads and treat different medical conditions, such as preventing organ rejection following organ transplantation.[6] This commentary will delve into the role amide protons have on improving the pharmacological properties of current and potential therapeutic peptides.

First and only FDA approved *N*-methylated therapeutic peptide, Cyclosporin A

Cyclosporin A (cyclosporin, cyclosporine), a metabolite produced by the fungus *Tolypocladium inflatum*, is a cyclic undecapeptide, cyclo-[MeBmt^{1}-Abu^{2}-MeGly^{3}-MeLeu^{4}-Val^{5}-MeLeu^{6}-Ala^{7}-D-Ala^{8}-MeLeu^{9}-MeLeu^{10}-MeVal^{11}], that was isolated by Sandoz (now Novartis) in the 1970's.[7] This peptide was found to be a powerful immunosuppressive agent and was introduced to the market in 1983 as Sandimmune® (Figure 1). The immunosuppressant properties of Cyclosporin A is used to prevent organ transplant rejection and treat a variety of immune-related disorders such as psoriasis and rheumatoid arthritis. Sandimmune® was initially offered as either an injectable or oral solution following its approval by the Food and Drug Administration (1983) and later offered as a liquid-filled capsule (1990; Neoral). Solution NMR and X-ray crystal structures revealed that the MeLeu^{9}-MeLeu^{10}-MeVal^{11}-MeBmt^{1}-Abu^{2} segment is vital for cyclosporin A to bind its target and exert its therapeutic effect. Nature's design of cyclosporin A has inspired many researchers to follow suit and conduct *N*-methyl

scanning to uncover vital amide proton interactions necessary for both inhibition and activation of specific targets, and for rationally designing peptide therapeutics.

N-methylation improves selectivity, stability and bioavailability

N-methyl scanning identifies amide protons of potential peptide therapeutics that are important for activity, bioavailability, stability and selectivity.[4-5, 8] Cilengitide, is a great example on how *N*-methylation improved selectivity (Figure 1). Horst Kessler and colleagues performed an *N*-methyl scan of a lead peptide antagonist, cyclo(R-G-D-f-V), and discovered a potent and selective antagonist, cyclo(R-G-D-f-*N*MeV) (Cilengitide), targeting the αvβ3, αvβ5 and α5β1 integrin receptors.[8] Cilengitide mainly antagonizes the αvβ3 integrin receptor but can also antagonize αvβ5 and α5β1 integrin receptors, all of which are vital cell adhesion proteins that were found to be overexpressed in many cancer cells and are involved in metastasis and proliferation. This peptide therapeutic, discovered by *N*-methyl scanning, was patented in collaboration with Merck for the treatment of glioblastoma, an aggressive cancer affecting the brain or spinal cord. Unfortunately, Cilengitide failed the European Organization for Research and Treatment of Cancer phase III trial and the Institute of Cancer Research phase II trial, resulting in Merck stopping its development in 2013. Iceni™ pharmaceuticals is currently evaluating the potential utilization of Cilengitide, in combination with proteasome inhibitors, to treat multiple myeloma.

Recently, *N*-methyl scanning was used to identify functional selectivity of the human urotensin II- receptor (UTR), a G protein-coupled receptor distributed throughout the body that is involved in cardiovascular regulation.[9-10] Functional selectivity, also known as bias agonism, is when multiple ligands can bind the same receptor but activate different signal transduction pathways based on the ligand identity. Paolo Grieco and colleagues conducted *N*-methyl scanning on two vasoactive cyclic peptide hormones, human urotensin II (*h*U-II) and urotensin II-related peptide (URP), along with the minimal active scaffold for UTR, fragmented urotensin II (U-II$_{4-11}$) (Figure 1). The researchers confirmed previous findings that the amide protons of Phe and Tyr are important for binding and activation, but discovered that the Lys and Trp residues are important for functional selectivity. *N*-methylation of URP Phe and Trp residues resulted in a significant loss of activation compared to the same modification in *h*U-II and U-II$_{4-11}$.[9] The researchers hypothesized that all ligands need a β-hairpin conformation to bind UTR and that functional selectivity between *h*U-II and URP might be due to differences in the orientation of the Lys and Tyr side chains. This is the first study to utilize *N*-methyl scanning as a tool to elucidate functional selectivity and highlight the potential of *N*-methyl scanning in unveiling the importance of specific amide protons in a peptide sequence.

The majority of therapeutic peptides target receptors and the native peptides that bind these receptors are usually short-lived due to the body's requirement to maintain homeostasis. The impressive arsenal of proteases and robust methods of peptide clearance also affect therapeutic peptides, presenting a challenge for medicinal chemists. Regioselective *N*-methylation of peptides is one method used to increase metabolic

stability against many proteases.[4-5] Anja Sandström and colleagues utilized *N*-methyl scanning to increase the stability of substance P 1–7 (SP_{1-7}) amide (NH_2-R-P-K-P-Q-Q-F-NH_2), an analog of the bioactive metabolite generated by the degradation of tachykinin substance P (SP) (Figure 1).[11] SP_{1-7} amide has been shown in different mice models to treat chronic neuropathic pain caused by spinal cord injury, spared nerve injury and diabetes. Sandström and colleagues found that *N*-methylation at Lys^3 and Gln^5 increases the peptide half-life in plasma from 6.4 min (SP_{1-7} amide) to over 180 min and 14.8 min, respectively. Unfortunately, these analogs were about half as potent as SP_{1-7} amide, but when compared to a commonly prescribed non-opioid neuropathic painkiller, gabapentin, these analogs were more potent and had no side effects.

Oral administration is the preferred method of administration of therapeutic drugs due to its convenience compared to other routes of administration.[12] Oral therapeutic peptides must overcome several barriers such as acidic conditions in the stomach, protein/peptide degrading gut enzymes, and intestinal permeability to become bioavailable by crossing the epithelial membranes and enter the bloodstream. Oral bioavailability refers to the proportion of an orally administered drug that cross the epithelial membrane and enter the bloodstream. Orally bioactive drugs must pass through the epithelial membrane either by a paracellular or transcellular pathway. The paracellular pathway is a passive method where the bioactive drug passes between the cells through tight junctions, while the transcellular pathway is through the cell and can occur either by passive diffusion, carrier-mediated active transport or vesicular uptake.[13] Peptide cyclization can be utilized to increase cell permeability, however, regioselective *N*-methylation has been shown to increase oral bioavailability. Scott Lokey and colleagues investigated whether β-branching, *N*-methylation or side chain size affect passive permeability of Sanguinamide A analogs, which are derivatives of the bioactive thiazole-containing cyclic peptide found in sea slugs (Figure 1). Using Caco-2 cells and parallel artificial membrane permeability assays (PAMPA), they found that *N*-methylation, in combination with aliphatic side chain extension, improve cell permeability better than β-branching. The recent discoveries that regioselective *N*-methylation of cyclic peptides can improve cell permeability are changing the way medicinal chemists develop orally bioavailable drugs because these compounds fail to follow Lipinski's rule of five (Ro5).

Medicinal chemists must take into account a compound's lipophilicity in their drug-discovery and drug-development phase, as it has been shown to affect compound's absorption, distribution, metabolism and excretion (ADME) properties.[14] Lipophilicity is a physicochemical property that defines a compound's affinity to a lipophilic environment and many metrics exist for determining compounds lipophilicity.[15] Recently, Lokey and colleagues have introduced a new metric, lipophilic permeability efficiency (LPE), that applies to "beyond rule of 5" (bRo5) molecules, which are compounds that fail to follow Ro5.[16] Lokey and colleagues defined LPE as a quantifiable unit that takes into account both membrane permeability and drug solubility as separable terms that can be used as a metric to predict a compound's passive permeability at a given aqueous solubility. This new metric can help medicinal chemists determine the necessary structural features a

compound needs for maximum passive cell permeability. Lokey and colleagues demonstrated the use of this new metric through *N*-methyl scanning of cyclic hexapeptides and cyclosporin A derivatives (natural and synthetic), but also determined the LPEs of the non-peptidic bRo5 molecules, rifamycins and erythronolides. They were able to show that the LPE values for various non-peptidic bRo5 molecules correlate with their cell permeability in Caco-2 cells. The researchers did acknowledge that LPE should be used as a general efficiency metric to assess compound's passive cell permeability performance because LPE does not always correlate with cell permeability.

Outlook on probing amide protons in peptide drug development

Probing the role of amide protons has demonstrated how they can affect the pharmacokinetic properties, including selectivity, stability and oral bioavailability of both natural and synthetic peptides. *N*-methyl scanning was traditionally utilized in structure-activity relationship studies to determine residues whose amide protons were important for activity and stability, however, recently *N*-methylation has been expanded to include functional selectivity. Furthermore, *N*-methyl scanning has been instrumental to the development of a new metric, LPE, that is aimed at assisting medicinal chemists rationally design bRo5 drugs with improved oral bioavailability. There has been an increased interest in understanding bRo5 molecules because about 20% of all new oral drugs fail to follow Lipinski's rule of 5.[17] We therefore expect that more attention will be given to the biophysical requirements for cell permeability of bRo5 molecules, and that *N*-methyl scanning will play a critical role in determining peptides structure-permeability relationships.[16]

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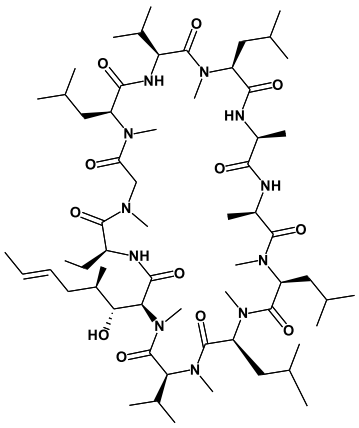
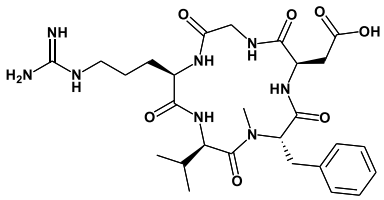
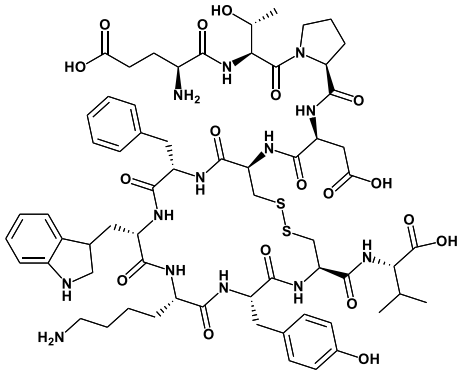


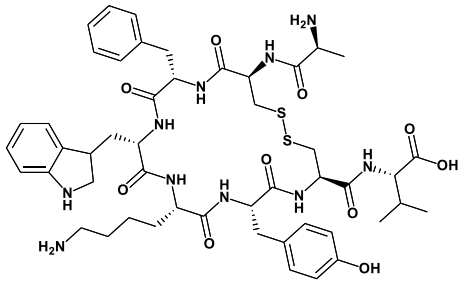
Cyclosporin A



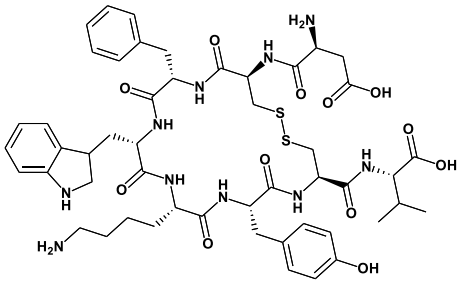
Cilengitide



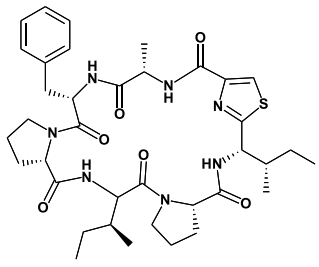
Human Urotensin-II



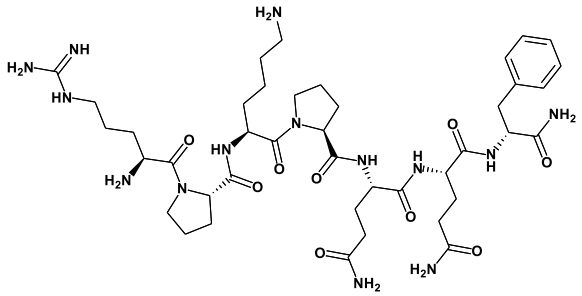
Human Urotensin-II related peptide



Fragmented Urotensin-II



Sanguinamide A



Substance P 1-7

Figure 1. Chemical structures of various therapeutic peptides discussed in this commentary.

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