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Improving Oral Bioavailability of Peptides by Multiple N-Methylation: Somatostatin Analogues**

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Dedicated to Ralph Hirschmann

Low bioavailability of peptides following oral administration is attributed to their inactivation in the gastro–intestinal tract through enhanced enzymatic degradation in the gut wall by a variety of peptidases expressed at the enterocytes brush border,[1] and to poor intestinal permeation.[2] In addition, the instability of peptides toward peptidases in the systemic blood circulation causes rapid elimination (i.e., short half-life). These factors limit the use of peptides as therapeutic agents in the clinical setting. Several strategies have been used to reduce enzymatic cleavage and uptake into the systemic blood circulation, including prodrug approaches, peptidomimetics, and structural modifications, such as covalent attachment of polyethylene glycol (PEG),[3] lipidation,[4] and chemical modifications, for example, cyclization,[5] D-amino acid substitution, and N-methylation.[6] Cyclic peptides show improved chemical stability and thereby display longer biological half-life compared to their linear counterparts.[7] Yet, additional modifications are required to generate peptides with enhanced enzymatic stability and improved oral bioavailability. One of the techniques suggested to improve the enzymatic stability of peptides is N-methylation.[8,9] We recently developed a simplified method which allows fast and efficient multiple N-methylation of peptides on solid support.[10] This simplified synthetic capability led us to study the influence of multiple N-methylation of the peptide backbone on its conformation and bioactivity.[11,12]

Inspired by the bioavailability of the highly N-methylated transplantation drug cyclosporin A, which can be administered orally although it violates all Lipinski's rules on oral bioavailability;[13] we assumed this bioavailability was a result of its multiple N-methylation together with cyclization. Thus, it is possible to overcome the above mentioned bioavailability drawbacks of peptides providing both the biological activity and the receptor selectivity by multiple N-methylation of cyclic peptides. Hence, we planned to screen a complete library of all the possible N-methylated analogues of the Veber–Hirschmann cyclic hexapeptide *cyclo*(-PFwKTF-) (**1**; Figure 1) which was reported to be selective towards sst2 and

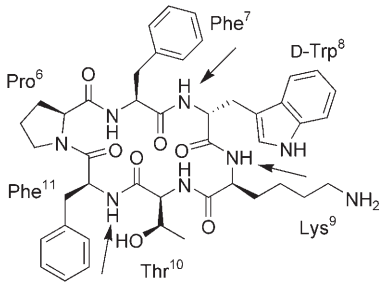

Figure 1. Veber–Hirschmann peptide *cyclo*(-PFwKTF-) (**1**). All the peptide bonds were N-methylated. The active analogues (see Table 1) resulted from the N-methylation of the amide bonds indicated by arrows. (The numbering corresponds to numbering in the native somatostatin).

sst5 subtypes of somatostatin receptor.[14] This approach gave rise to 31 different N-methylated analogues (2^5-1). Octreotide (Sandostatin),[15] a synthetic somatostatin analogue, is currently used as a drug for the therapy of acromegaly and for the symptomatic treatment of intestinal endocrine tumors; however, because of its low oral bioavailability it is administered parenterally. We envisioned that multiple N-methylation could transform the cyclic hexapeptide *cyclo*(-PFwKTF-) into a bioactive analogue that would be orally available.

The library of 30 (the penta N-methylated analogues could not be synthesized successfully) N-methylated peptides was synthesized on solid support (linear peptides) and cyclization was carried out in solution. Though the synthesis of linear peptides was straightforward, cyclization proved to be a crucial step. All the head-to-tail cyclizations were carried out at the free N-terminal end with the solid-base method

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(dipehenylphosphorylazide (DPPA)/$NaHCO_3$).[16] The cyclization yield was also dependent on the sequence of the linear precursor. As the conformation of the stem peptide *cyclo*-(-PFwKTF-) has a βII′ turn about D-Trp[8] and Lys[9] and a βVI turn about Phe[11] and Pro[6][17] Lys[9] was chosen as the C-terminal amino acid to be linked to the tritylchloridepolystyrene (TCP) resin in all the cases where Thr[10] was non N-methylated. Thus, during cyclization, though the linear peptide exhibits a dynamic nature in solution, it will always prefer a turn structure, this brings the N-terminal and C-terminal ends into close proximity to enhance cyclization. On scaling up the synthesis of analogue **8**, the cyclization with HATU/HOBt and collidine gave excellent results.[18]

In vitro screening of the N-methylated cyclic hexapeptide library by binding to all the human SRIF receptor subtypes (hsst1–5), gave only seven analogues showing affinity similar to the parent peptide (that is, in the nanomolar range to receptor subtypes hsst2 and hsst5; Table 1). These seven analogues were administered to rats to check their uptake into blood and interestingly, only **1** and **8** showed significant

Table 1: pK_d values for the N-methylated sublibrary (peptides **1**–**8**), compared to Octreotide, towards hsst2 and hsst5 receptors expressed in CCL-39 cells and measured by radioligand binding assays with [^{125}I]LTT-SRIF28 as radioligand.[19]

Peptide	N-methylated amino acid	hsst 2 (pK_d)	hsst 5 (pK_d)
Octreotide	–	9.18	7.71
1	–	8.01	7.82
2	Lys[9]	8.60	8.19
3	Phe[11]	7.93	8.28
4	D-Trp[8]	7.61	7.87
5	Lys[9], Phe[11]	7.96	7.39
6	D-Trp[8], Lys[9]	7.60	7.19
7	D-Trp[8], Phe[11]	7.16	7.47
8	D-Trp[8], Lys[9], Phe[11]	7.21	7.22

uptake into the blood stream with a plasma concentration of 242 ng mL^{-1} after 30 min and 151 ng mL^{-1} after 1 h for **8** and 158 ng mL^{-1} and 38 ng mL^{-1} at the same time points for parent peptide **1**. Thus, we decided to characterize the detailed pharmacology including the mode of transport of **1** and **8**.

The enzymatic stability of the peptide sublibrary was evaluated in rat serum. No significant degradation was observed for any of the peptides after 7 h incubation (see Supporting Information). In addition to serum stability, analogues **1** and **8** were evaluated for their stability in the gastro–intestinal (GI) tract using enzymes isolated from the brush border (Brush Border Membrane Vesicles, BBMVs). These enzymes include a variety of peptidases which participate in the digestion of peptides and proteins in the gut wall,[20] thus they can serve as an in vitro tool to evaluate peptide stability in the GI tract. As can be seen in Figure 2, the non-methylated stem peptide **1**, was degraded following exposure to intestinal enzymes. After 30 and 90 minutes incubation, 15% and 25%, respectively, of the peptide was degraded. In comparison, analogue **8** was found to be completely stable to enzymatic degradation under the assay conditions.

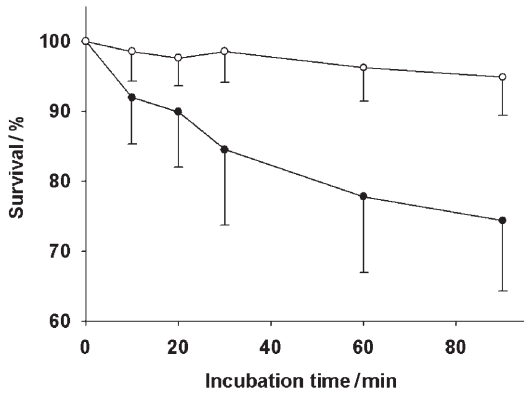

Figure 2. Stability of peptides **1** (●) and **8** (○) in brush border membrane vesicles (BBMVs). The tested molecules were mixed with BBMVs and incubated in 37 °C for 90 minutes, $n = 4$. Data are expressed as the mean ± SEM (standard error of the mean value). Statistical analysis gave a "student's t test" value of $p < 0.05$.

The peptides were evaluated for their intestinal permeability using the Caco-2 in vitro model and compared to mannitol, a marker for paracellular permeability. The calculated permeability coefficients (*P* apparent, P_{app}) of the tested compounds are depicted in Figure 3. The permeability of analogues **1**–**4**, **6**, and **7** was lower than 1×10^6 cm s^{-1}. Analogue **5** was found to be relatively more permeable

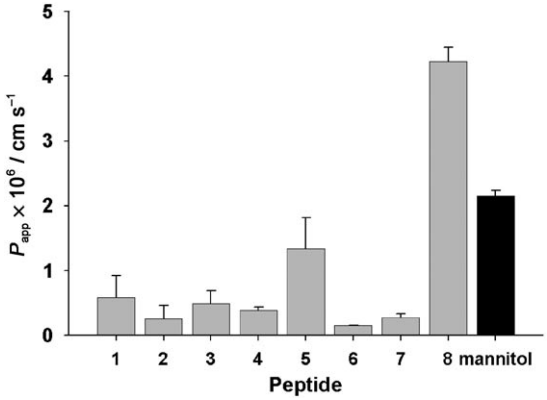

Figure 3. Permeability coefficient P_{app} of the eight biologically active analogues, compared to mannitol (paracellular marker), in the Caco-2 monolayer.

(1.8×10^6 cm s^{-1}), interestingly analogue **8** was found to have the highest P_{app} value (4×10^6 cm s^{-1}) exceeding that of mannitol.

To evaluate the possible involvement of active transport mechanism in the permeability process, **8** was evaluated for its permeability from the apical to the basolateral side (A to B) and in the reverse direction (basolateral to apical, B to A; see Supporting Information). The permeability rate was found to be identical for both directions suggesting that no active transport is involved in the permeability of **8**.

A novel colorimetric assay[21] was used to assess whether the peptides interact with a bilayer liposome which functions as a model of the cell membrane. When comparing a set of

analogues that have the same number of N-Me groups (Figure 4), there were two analogues (**6** and **7**) with enhanced interaction with the liposome (>85%) while **5** interacted poorly with the vesicle membrane (<20%). Analogue **1**

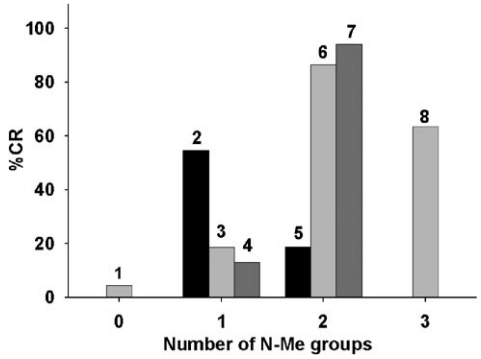

Figure 4. The effect of N-Me position on the interaction with a liposomal model of the cell membrane (% cell response (CR)). Analogues with identical numbers of N-Me groups (1 methyl group: peptides **2**–**4**, 2 methyl groups: peptides **5**–**7**) were screened together with **1** and **8** for their interaction with the bilayer liposomal model.

showed negligible interaction with the membrane, indicating that it is unable to penetrate through the model bilayer, while **8** showed a significant interaction with the membrane. These results suggest that, although the increase in the lipophilic nature of the analogues caused by additional N-Me groups, as shown by *c*log*P* values, results in an increased interaction with the membrane, there is no linear correlation between increasing numbers of N-Me groups and enhanced interaction with the membrane (measured by a color reaction). In other words, the data show the importance of the N-Me group position; different degrees of interaction occur with peptides having the same number of N-Me groups but at different positions (Figure 4).

The pharmacokinetic parameters of **1** and **8** are significantly different, they show a fivefold difference in the elimination half-life ((15.5 ± 2) and (74 ± 6) min, respectively) and a tenfold difference in the volume of distribution at steady state (V_{ss}, (0.3 ± 0.1) and $(3.7\pm1.3)\,L\,kg^{-1}$, respectively). Additional distinctive characteristics were revealed following per oral (p.o.) administration. Following administration of **1** and **8** by oral gavage, at a dose which is one order of magnitude higher that the intravenous (i.v.) dose (i.e., $10\,mg\,kg^{-1}$ vs. $1\,mg\,kg^{-1}$), peptide **1** could be detected only in one rat (out of 4), therefore a pharmacokinetic profile following oral administration could not be depicted. On the other hand, using the same dose for **8** provided a full pharmacokinetic profile of concentration versus time in blood (Figure 5). The calculated absolute oral bioavailability of **8** was 9.9%.

The impact of N-methylation was evaluated in two different biological media, blood and intestinal wall (BBMVs) that contain different types of peptide degrading enzymes, and are very relevant in dictating the pharmacokinetic fate of the bioactive peptide in the body. The peptides were found to be stable in rat serum, a result which was

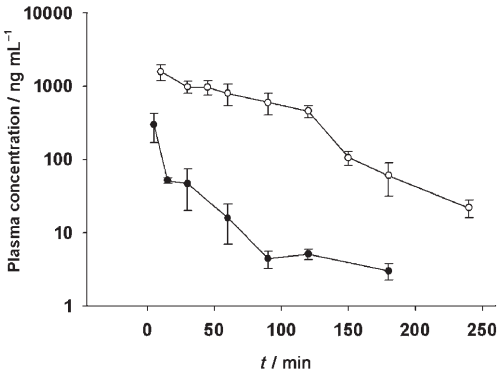

Figure 5. Plasma concentration–time profiles (Mean ± SEM) following oral (○) and intravenous (●) administration of 10 $mg\,kg^{-1}$ and 1 $mg\,kg^{-1}$, respectively, peptide **8**, $n=4$.

expected as all the peptides in the sublibrary are small and cyclic[22,23] and the diversity of enzymes in the plasma is limited. On the other hand, comparing the two extreme cases of unmethylated peptide **1** with trimethylated peptide **8** in purified brush border enzymes (Figure 3) revealed the significant contribution of multiple N-methylation to the stability of peptide **8**. This finding may explain the high stability of the drug cyclosporine A, in human serum[24] as indicated by its relatively long biological half-life, 6.2 h (in man).[25] Cyclosporine A similarly to **8**, is cyclic, multi N-methylated, and also exhibits metabolic stability against the harsh peptidase activity in the intestinal wall. This stability against peptidases degradation is most probably attributed to the synergistic impact of cyclization together with multiple N-methylation.

An additional factor that limits the oral bioavailability of peptides is their low permeation through the intestinal wall. In the case where there is no active transport involved in the peptide absorption, they may penetrate across the enterocytes by passive diffusion mechanisms, either through the membrane (transcellular) or between the enterocytes (i.e., the paracellular pathway). Whereas hydrophilic molecules tend to be absorbed across the intestinal wall by the paracellular route, lipophilic compounds can permeate transcellularly.[26] This route provides extensive flux, in comparison to the paracellular route, mainly as a result of the significantly larger surface area.[27] It was suggested that by increasing the lipophilicity of peptides, the permeability could be shifted to transcellular absorption.[28] An approach to achieve increased lipophilicity is by multiple N-methylation. Thus, this structural modification could provide a possible shift from paracellular towards transcellular absorption mechanism. According to our findings in the Caco-2 permeability model, all the tested peptides, except for peptide **8** were found to have P_{app} values which are lower or comparable to the P_{app} of mannitol, a marker for paracellular transport. Nevertheless, the permeability coefficient of **8** was significantly higher (68% increase) than the non N-methylated **1**, suggesting that multiple N-methylation improved the intestinal permeability, even in the aqueous media of the tight-junction pores that enable paracellular transport. The possibility of increased permeation through an active transport mechanism of **8** was ruled out by

the finding that there was no observed difference in the permeation rate of the peptide when measured from apical to basolateral side and vice versa (see Supporting Information). To eliminate the possibility of model-dependent results, additional in vitro methods including MDCK cells[29] and side-by-side diffusion chamber[30] were used to verify the transport characteristics of **8**. The permeability coefficient found in these models was in the range of paracellular transport (data not shown).

Analogues **1** and **8** were evaluated for their oral bioavailability in vivo following i.v. and p.o. administration to rats (Figure 5). While the Veber–Hirschmann peptide was not orally available, the absolute oral bioavailability of the N-methylated peptide **8** was about 10% of the administered dose. In addition, changes were also found in additional pharmacokinetic parameters. The enhanced volume of distribution of **8** compared to **1** (3.7 and 0.3 $L\,kg^{-1}$, respectively) suggests that while the distribution of **1** is limited to the blood and the interstitial fluid, **8** can interact with biological membranes. A difference was also found in the plasma half-life of **1** and **8** which may have resulted from reduction of proteolytic digestion or hepatic and/or renal clearance. The transcellular transport includes an interaction of the molecule with the hydrophobic membrane followed by crossing the membranes (i.e., the apical and basolateral membranes) to reach the blood circulation. Indeed, an increase in the interaction of the N-methylated peptides with a model of the cell membrane was observed for the N-methylated peptides (Figure 4). Yet, this liposomal model is limited to evaluate the interaction with the membrane, this interaction is a mandatory but not exclusive condition to cross the membrane. The enhanced interaction of **8**, observed in the membrane vesicle liposome model may clarify the discrepancy between the in vitro permeability models which show limited absorption and the enhanced volume of distribution, compared to **1**. The fact that peptides with identical numbers of N-methyl groups hold different degree of interaction with the liposomal membrane model suggest that there are additional factors, including conformation, that affect the interaction.

It is interesting to note that in all of these seven bioactive analogues of the sublibrary, the βII′ and the βVI turn are conserved even in the tris N-methyl compound **8** (Figure 6), which corroborates with the earlier results that these two turns maintain the peptide in the bioactive conformation.[17]

In general, we observe an enhancement in the binding affinity when the molecule contains $MeLys^9$ and a reduction with Me-D-Trp^8 or $MePhe^{11}$, this subtle modulation in the activity could be understood by analyzing the conformations of these analogues. Goodman et al. suggested the bent conformation of the peptide as the bioactive conformation, which is stabilized by the two γ turns about Phe^7 and Thr^{10}.[31] This bent conformation results in the deep insertion of the Lys^9, D-Trp^8, and Phe^{11} in the receptor; N-methylation of Lys^9 enhances the stability of the bent conformation by reducing flexibility about the βII′ turn tuning the peptide into a more potent analogue. N-Methylation at either D-Trp^8 or Phe^{11} decreases the activity owing to the loss of the stabilizing γ turns; however, it is interesting to note that the Me-D-Trp^8 analogue is less active than $MePhe^{11}$. This result gives an indication of the importance of the spatial orientation of Phe^{11}. Although there is a loss in the bent conformation by N-methylation of Phe^{11} which results in the deep burying of the phenyl ring, it retains an activity comparable to the stem peptide. These results suggest that multiple N-methylation can also be useful in elucidating fine details of the bioactive conformation.[12]

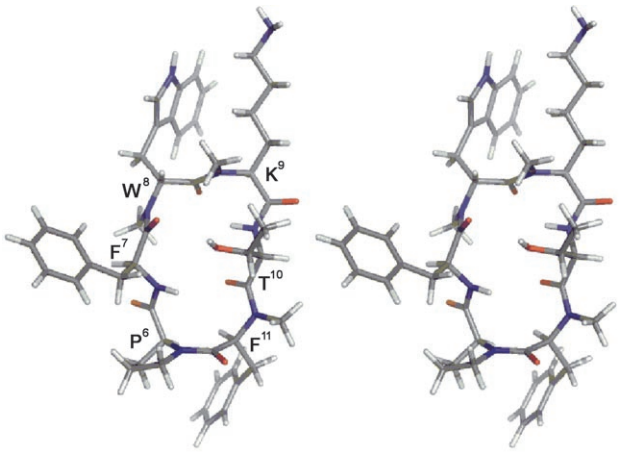

Figure 6. Stereoview of *cyclo*(-PFMewMeKTMeF-) (**8**).determined by NMR spectroscopy and molecular dynamics calculations (see Supporting Information); O red, N blue.

To determine the importance of the Phe^{11} for bioactivity, we have synthesized the epimeric analogue of **8**, *cyclo*-(-PFMewMeKTMef-) in which the $MePhe^{11}$ is substituted by the enantiomeric D-$MePhe^{11}$. In contrast to **8** this peptide exhibits a *trans* peptide bond resulting in a βII turn instead of a βVI turn about Phe^{11} and Pro^6, causing the loss in deep burying of the phenyl ring and consequently the loss of activity. The membrane permeability of this peptide was also greatly reduced when compared with that of peptide **8**. From this result we conclude that Phe^{11} and its surroundings are important, not only in maintaining the activity of the peptide, but also in maintaining the permeability profile of a peptide.

In summary we have characterized the effect of multiple N-methylation on the intestinal permeability and enzymatic stability of somatostatin analogues. Improving these parameters is a key factor in enhancing the oral bioavailability of peptides. We show that multiple N-methylation of a cyclic peptide improved its oral bioavailability without modifying its biological activity and selectivity. This finding is a step towards the development of peptide based therapeutics. Thus, multiple N-methylation could be a simple way to achieve oral bioavailability of peptidic drugs.

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