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Fine-tuning the physicochemical properties of peptide-based blood–brain barrier shuttles

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ABSTRACT

N-methylation is a powerful method to modify the physicochemical properties of peptides. We previously found that a fully *N*-methylated tetrapeptide, Ac-(*N*-MePhe)$_4$-CONH$_2$, was more lipophilic than its non-methylated analog Ac-(Phe)$_4$-CONH$_2$. In addition, the former crossed artificial and cell membranes while the latter did not. Here we sought to optimize the physicochemical properties of peptides and address how the number and position of *N*-methylated amino acids affect these properties. To this end, 15 analogs of Ac-(Phe)$_4$-CONH$_2$ were designed and synthesized in solid-phase. The solubility of the peptides in water and their lipophilicity, as measured by ultra performance liquid chromatography (UPLC) retention times, were determined. To study the permeability of the peptides, the Parallel Artificial Membrane Permeability Assay (PAMPA) was used as an *in vitro* model of the blood–brain barrier (BBB). Contrary to the parent peptide, the 15 analogs crossed the artificial membrane, thereby showing that *N*-methylation improved permeability. We also found that *N*-methylation enhanced lipophilicity but decreased the water solubility of peptides. Our results showed that both the number and position of *N*-methylated residues are important factors governing the physicochemical properties of peptides. There was no correlation between the number of *N*-methylated amide bonds and any of the properties measured. However, for the peptides consecutively *N*-methylated from the N-terminus to the C-terminus (p1, p5, p11, p12 and p16), lipophilicity correlated well with the number of *N*-methylated amide bonds and the permeability of the peptides. Moreover, the peptides were non-toxic to HEK293T cells, as determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.

1. Introduction

The blood–brain barrier (BBB) is the body's natural defense against potential toxic compounds circulating in the blood. At the same time, the BBB allows certain compounds required for brain function to enter this organ by means of various mechanisms, including active transporters and passive diffusion. However, the treatment of brain disorders is hindered by the presence of the BBB, since this barrier limits the transport of drugs from the blood to the brain.[1–3]

Several invasive and non-invasive approaches have been used to overcome the BBB and deliver drugs to the brain. The latter include vector-mediated strategies, in which compounds such as antibodies,[4–6] nanoparticles,[7–10] sugars,[11–13] amino acids[14,15] and peptides[16–24] are used as shuttles to carry drugs (that cannot reach the brain unaided) across the BBB.

In recent decades, peptides have emerged as promising pharmaceutical agents or drug carriers for the treatment of brain disorders. Many peptides cross the BBB by passive diffusion.[25–30] Properties such as lipophilicity and molecular weight are the major factors that govern the passage of compounds across this barrier. In addition, peptide size and the type of amino acids can also affect peptide permeation through biological barriers.[31,32]

The *N*-methylation of peptides increases their lipophilicity and permeation in *in vivo* and *in vitro* models of the BBB.[33] Chikhale et al.[34] and Burton et al.[35] attributed the enhanced transport of *N*-methylated peptides through the BBB to a decrease in number of hydrogen bonds that the peptides can form. Thansandote et al.[36] showed that mono-, di- and tri-*N*-methylated analogs of a cyclic

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hexapeptide exhibited greater lipophilicity and permeability values than the non-methylated version. However, when the number of *N*-methylated amino acids increased, the aqueous solubility of the peptides decreased. Ovadia et al.[37] reported that the permeability of a family of cyclic hexapeptides across cell membrane varied in function of the number and position of *N*-methylated residues. These findings were confirmed by Wang et al.[38], who also observed that the degree and position of *N*-methylation had a remarkable impact on ability of a different series of cyclic hexapeptides to transport across cell membranes. Interestingly, Bose et al.[39] found that *N*-methylation enhanced both the lipophilicity and water solubility of the peptides. We also previously reported that fully *N*-methylated peptides are more lipophilic and water-soluble than the non-methylated parent peptides. Moreover, the fully *N*-methylated peptides are able to cross artificial membranes while the non-methylated peptides show no permeability.[40]

Our research has thus far largely been limited to peptides containing only *N*-methylated amino acids.[40–42] We now wish to explore the relationship between the position and number of *N*-methylated amino acids and peptide bioavailability. Hence, peptides Ac-$(Phe)_4$-$CONH_2$ and Ac-$(N\text{-MePhe})_4$-$CONH_2$ were used as scaffolds. We previously showed that the former does not pass through the artificial membrane while the latter does. In addition, the peptide Ac-$(N\text{-MePhe})_4$-$CONH_2$ was tested as a potential BBB-shuttle. In this regard, it has demonstrated ability to carry several non-permeating drugs through artificial and cell membranes.[40,41] Based on these observations, and in order to fine-tune BBB-shuttle permeability and study the effect of the degree and position of *N*-methylated groups on peptide permeability, we designed and synthesized a family of 16 peptides with different numbers and positions of *N*-methylated amino acids in their sequences. The lipophilicity, aqueous solubility and permeability of the resulting peptides were then evaluated. *N*-methylation improved lipophilicity and permeability but decreased aqueous solubility. In addition, both the position of *N*-methylated groups and the degree of *N*-methylation had an impact on the physicochemical properties of the peptides.

2. Results and discussion

In order to optimize the permeability of a potential BBB-shuttle peptide, we previously studied the relationship between PAMPA permeability and the number of *N*-methylphenylalanine (*N*-MePhe) residues on peptide length. In a series of *N*-MePhe oligomers, increasing peptide chain length enhanced PAMPA permeability up to the peptide with 4*N*-MePhe residues, namely Ac-$(N\text{-MePhe})_4$-$CONH_2$. However, for the peptide with more than 4 of these residues, PAMPA permeability dropped markedly (Fig. 1a). We also found that the peptide with 4 *N*-MePhe residues and longer peptides were too lipophilic.[40] It is possible that overly lipophilic peptides get trapped in the membrane and consequently show lower permeability. On the basis of those findings, we synthesized new analogs of Ac-$(N\text{-MePhe})_4$-$CONH_2$ in order to optimize the lipophilicity of the peptide and improve its permeability. To this end, a library of 16 tetrapeptides, which differ only in the number and position of *N*-MePhe residues, was designed and synthesized using a solid-phase peptide synthesis (SPPS) technique and characterized by ultra performance liquid chromatography (UPLC), ultra performance liquid chromatography–mass spectrometry (UPLC-MS) and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF). The lipophilicity, water solubility and PAMPA permeability of the peptides was then studied. Our results also showed how lipophilicity, aqueous solubility and permeability of our peptides are influenced by changing the number and position of *N*-MePhe residues.

2.1. Lipophilicity

The relative lipophilicity of peptides can be determined on a reverse-phase high performance liquid chromatograph (HPLC) or UPLC column on the basis of their retention time (t_R).[39,42–44] The retention times of our peptides in a UPLC column were used as a measure of their relative lipophilicity. The lipophilicity values differed between peptides. In theory, the addition of each methyl group to a peptide bond causes an increase in the lipophilicity of the molecule as a result of a decrease in the number of hydrogen bonds that it can form.[34,35] However, none of the peptides followed this pattern. There was a weak correlation (r = 0.543) between peptide lipophilicity and number of *N*-methylated amide bonds (Fig. 1b). For example, p9 and p15 with 2 and 3 *N*-methylated amide bonds, respectively, exhibited lower lipophilicity than p2, p3, p4 and p5, which had only one *N*-methylated amide bond (Table 1). These findings are in line with previous reported results. Burton et al.[35] found that, for a peptide with 3 Phe residues, the analog with 2 *N*-methylated amide bonds was less lipophilic than the peptide with only one *N*-methylated amide bond. For a hexapeptide corresponding to residues 16–21 of β-amyloid peptide (Aβ), Bose et al.[39] also found that the analog with 5 *N*-methylated amide bonds showed lower lipophilicity compared to that of the peptide with 3. The peptides with the same number of *N*-MePhe residues but at different positions in their structures (for example, p2 and p4, p6 and p8, p12 and p15) showed distinctive lipophilicity. We observed good correlation (r = 0.913) between lipophilicity and

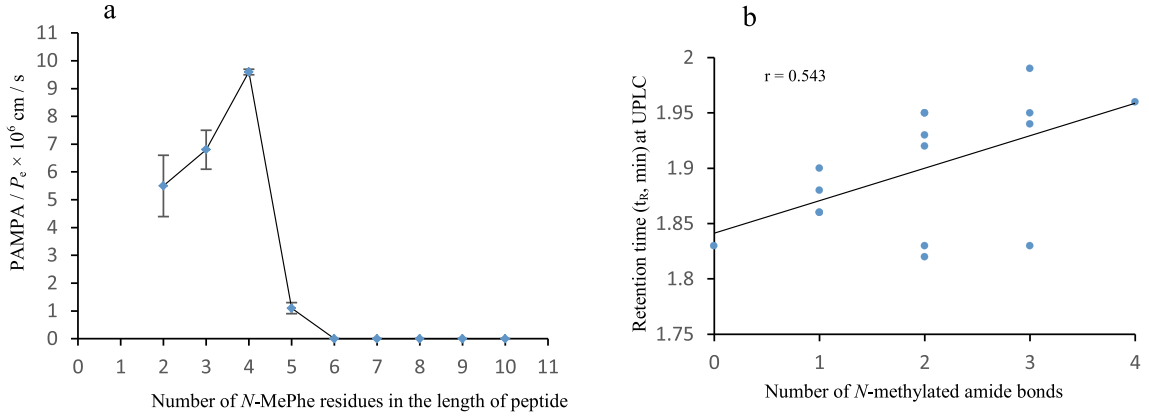

Fig. 1. a) Relationship between PAMPA P_e and peptide length. Data are expressed as the mean (n = 3) ± SD. From ref 40. b) Correlation between UPLC t_R of 16 peptides and number of *N*-methylated amide bonds in their sequences.