Bioorganic & Medicinal Chemistry 18 (2010) 5896–5902

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

In vitro ADMET and physicochemical investigations of poly-N-methylated peptides designed to inhibit Aβ aggregation

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ARTICLE INFO

Article history:
Received 22 April 2010
Revised 24 June 2010
Accepted 28 June 2010
Available online 1 July 2010

Keywords:
Alzheimer's disease
Amyloid β peptide
N-Methylated peptide
ADMET

ABSTRACT

N-Methylation is a common strategy for improving oral bioavailability of peptide-based lead structures. Herein, we present a detailed study on how the degree of N-methylation affects the absorption–distribution–metabolism–excretion–toxicity (ADMET) properties such as solubility, membrane transport, proteolytic stability, and general cell toxicity of the investigated peptides. As representative structures we chose hexapeptides **1**–**8**. These peptides, corresponding to N-methylated analogues of residues 16–21 and 32–37 of the Aβ-peptide, pathological hallmark of Alzheimer's disease (AD), have previously been shown to inhibit aggregation of Aβ fibrils in vitro. This study suggests that poly-N-methylated peptides are non-toxic and have enhanced proteolytic stability over their non-methylated analogues. Furthermore, solubility in aqueous solution is seen to increase with increased degree of N-methylation, while membrane transport was found to be low for all investigated hexapeptides. The present results, together with those reported in the literature, suggest that poly-N-methylated peptides, especially shorter or equal to six residues, can be suitable candidates for drug design.

1. Introduction

The advent of more efficient methods for synthesis and improved screening processes of new compounds are contributing to a day by day increase of the number of chemical leads identified in the drug discovery phase.[1,2] It is evident from the research of last decades that compounds having good biological activity may fail to become good drug candidates due to low bioavailability after oral administration. Selection of drug candidates with adequate absorption characteristics, greater endurance at enzyme insult in the GI (gastrointestinal) tract, and no or tolerable cellular toxicity should increase the probability of success in the development phase. Proteins and peptides have become promising scaffolds for modern drug discovery.[3,4] The chief advantage of peptides to serve as leads in drug discovery is the possibility of rational design from endogenous ligands, their high specificity, and a wealth of highly developed methods for analyzing their mode of actions.[5,6] However, there are also prominent challenges before peptides and proteins can be used as drugs. Many peptides suffer from low bioavailability due to proteolysis and poor membrane permeability, which limit their application as drugs due to unfavorable pharmacokinetics.[7] Over the years medicinal chemists have developed an array of strategies to confront these problems, such as incorporation of peptide bond isosters, retro-inverso peptides, peptoids, peptidomimetics, structural modifications such as covalent attachment of poly ethylene glycol (PEG), lipidation and chemical modifications.[8–11] N-Methylation of the amide bond is one of the chemical modification approaches, which is very efficient for increasing the proteolytic stability of peptides. N-Methylated peptides were shown to have improved pharmacological properties such as lipophilicity, proteolytic stability, bioavailability, and conformational rigidity.[12–14] Additionally, incorporation of *N*-methyl amino acids have been reported to lead to enhanced potency and new receptor subtype selectivity; for example, cyclic RGD peptides and dermorphin analogs have been shown to be more potent following mono N-methylation.[15,16] N-Methylation scanning is an

Abbreviations: ADMET, absorption–distribution–metabolism–excretion–toxicity; AD, Alzheimer's disease; Aβ, amyloid β-peptides; BBB, blood–brain barrier; BSA, bovine serum albumin; BTC, bis(trichloromethyl)carbonate; DMPK, drug metabolism and pharmacokinetics; ELSD, evaporative light-scattering detection; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyltetrazolium bromide; QC, quality control.

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doi:10.1016/j.bmc.2010.06.087

approach where a library of all possible N-methylated peptide analogs, based on a sequence of a linear or cyclic bioactive peptide, is synthesized and screened to select an active N-methylated analog. This approach has led to the discovery of a metabolically stable, intestinally permeable somatostatin analog and a RGD analog with new receptor selectivity.[13,17,18]

Similarly, peptides containing single or multiple N-methylated amino acids have been identified as especially promising agents to block protein–protein interactions involving β-sheet rich interactions, as illustrated by inhibitors for amyloid β peptide (Aβ)[19–22] and amylin[23–28] fibrillation. The senile plaques seen in the brains of Alzheimer's disease (AD) patients are composed of oligomers of the 38–43 amino acid long Aβ,[29,30] while fibrillar aggregates of amylin in the Islets of Langerhans is representative for Diabetes type II.[31–33] Consequently, prevention of Aβ and amylin aggregation has emerged as potential goals in the therapy and prevention of AD and diabetes, and various β-sheet disrupting peptides have been reported to preclude the aggregation of Aβ[34–39] and amylin.[40–42]

Although, the N-methylation approach is recognized as a useful method for enhancing selectivity and improving pharmacological properties of peptides, there are few studies that completely addressed the basic changes in physicochemical and in vitro DMPK-like properties (drug metabolism and pharmacokinetics) brought about by modifying a peptide sequence by multiple N-methylations. Gordon et al. studied inhibitors based on the 16–22 region of Aβ, and showed that the aggregation inhibition potential, as well as their protease resistance, was optimal when N-methylated residues were incorporated in every alternate position of the peptide.[43] Adessi et al. assessed the ADMET properties of a series of anti-amyloid pentapeptides, including a set of N-methylated derivates; their study showed that a pentapeptide with one *N*-Me amide bond was able to cross the blood–brain barrier (BBB) after iv dosing in vivo.[44] The last paper we are aware of on this topic is the recent paper by Giralt and co-workers that aimed to develop a multiple N-methylated peptide sequence that could act as a 'Trojan horse' for BBB-delivery.[45] Their detailed investigations revealed an interesting structure activity relationship for hydrophobic N-methylated peptide sequences of varying chain lengths, all containing a repeated *N*-Me phenylalanine motif. In this account, we aim to complement the above-mentioned studies by investigating N-methylated peptides with non-specific sequence motifs and with a mixed degree of hydrophobicity in order to increase the general understanding of physicochemical and in vitro ADMET properties for this widely used class of lead compounds.

2. Results and discussion

As representative peptide structures two sets of peptides were selected which are known to block aggregation of the related Aβ-peptide—postulated to be of importance for Alzheimer' disease.[34,46] Our group recently reported that peptides **5**–**8** (Fig. 1), corresponding to the residues 32–37 of Aβ, and containing a variable number of N-methylated amino acid residues, have the ability to reduce Aβ induced toxicity.[22] Gordon et al. discussed the potency of the series of N-methylated peptides derived from 16–22 of Aβ,[43] which resembled our peptides **1**–**4** (shorter by one amino acid). Both series of peptides are capable of inhibiting Aβ fibril formation, with the non-methylated peptide **1** being the most efficient among the peptides **1**–**4** corresponding to the 16–21 region, while the poly-N-methylated peptide **8** was the best inhibitor of Aβ fibril formation among the peptides corresponding to the 32–37 region.

2.1. Solubility results

Solubility is a key physicochemical property of drug substances, as high solubility is a prerequisite to get a rapid absorption and a high bioavailability of the compound. In order to evaluate the effect of N-methylation on the solubility of the peptides, we measured the experimental solubility through the classical shake-flask method. This protocol generates a thermodynamic equilibrium soluble state, that is, the saturation solubility of a compound in equilibrium with an excess of non-dissolved substance.[47]

By inspection of the data in Table 1, it can be seen that N-methylation increases the solubility ~100–1000-fold compared to the non-methylated peptide **1** and **5**. Several of the N-methylated peptides, that is, peptides **3**, **6**, and **8**, exceeded the upper limit (2.5 mM) of the method. Table 1 demonstrates that the dramatic increase in solubility observed is seen already with two, out of seven amide bonds, being N-methylated, namely peptides **2** and **6** (compared to peptides **1** and **5**, respectively). The main reason for this effect is most likely the decrease in intermolecular hydrogen-bonding interactions between peptide strands in the solid state, as a consequence of N-methylation. It is also likely that the peptide bond of N-methylated peptides is experiencing an increase in polar character due to the positive inductive effect of the methyl group. Combination of these two factors could explain the phenomenal increase in solubility of peptides in aqueous solution.

2.2. Lipophilicity

Lipophilicity, as determined by calculated (*C*log *P*), or experimental chromatographic estimated lipophilicity (*E*log *D*), octanol–water partition (log *P*), or distribution (log *D*) constant, is expected to reflect the physiological distribution of the neutral (log *P*) or the charged (log *D*) species within the body. log *P*/*D* values are thus important descriptors of absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties.[47–49] Lipophilicity is also an important descriptor of blood–brain barrier (BBB) permeability,[50,51] and also plays a dominant role in toxicity predictions.[52]

The lipophilicity of peptides **1**–**8** was determined with a chromatographic method that estimates log *D*-values using UPLC–UV/MS, and thus provides an approximate, so-called estimated log *D* (*E*log *D*) of the substance.[53] The results, shown in Table 1, reveal that for peptides **5**–**8** the estimated lipophilicity increased with the degree of N-methylation, as might be expected based on the structural modification of replacing hydrogen atoms with methyl-groups. For peptides **1**–**4**, which contains a positively charged lysine residue, this effect is somewhat less pronounced. Here, the lipophilicity increased when going from none to two N-methyl amide bonds (cf. peptides **1** and **2**), but then stagnated and even declined slightly, upon incorporation of additional *N*-methyl amino acids (i.e., three *N*-Me amide bonds in **3** and five *N*-Me amide bonds in **4**). This observed difference between the two peptide families **1**–**4** and **5**–**8** most likely reflect the presence of a charged, that is, hydrophilic, residue in the peptides **1**–**4**, which effectively counterbalances the increased lipophilicity imposed by the additional *N*-methyl bonds. This explanation is supported by the calculated *C*log *P* and ACD log *P* values also shown in Table 1. These calculations, which reflect the lipophilicity of the neutral form of the peptides, qualitatively predicts an increased lipophilicity upon replacing the N–H functionality with increasing number of *N*-Me groups; neither of the methods are especially good as predicting the experimental *E*log *D* values quantitatively, as seen for peptides **5**–**8** that lack ionizable groups.

It is fascinating that N-methylation of peptides is able to both increase the aqueous solubility as well as the lipophilicity of the peptide in relation to the non-methylated peptide.

2.3. Stability towards enzyme degradation

Native peptides can rarely be applied as pharmacologically active compounds in a biological system due to rapid degradation

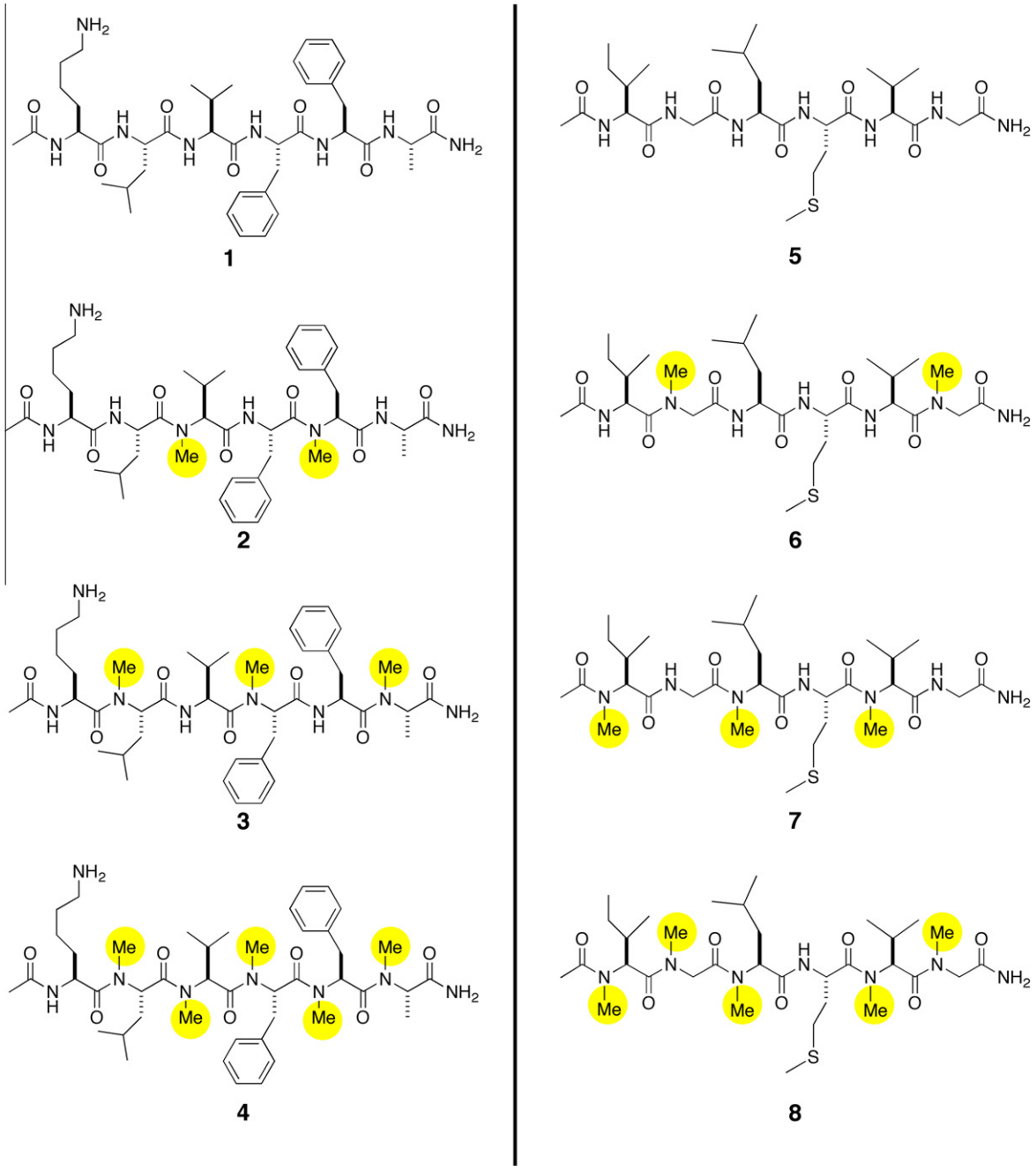

Figure 1. Structures of peptides **1**–**8**.

by proteases. In an effort to address the postulated enhanced proteolytic stability of N-methylated peptides,[12–14,19–28,43,44] we subjected our peptides to pronase. Pronase is a non-specific protease. Its proteolytic activity extends to both denatured and native proteins, which in general are broken down into individual amino acids. This property is attributable to the composition of the preparation, which comprises various types of endopeptidase and exopeptidase. Typically, neutral protease, chymotrypsin, trypsin, carboxypeptidase, and aminopeptidase are present, together with neutral and alkaline phosphatases which make it an aggressive cocktail of enzymes of different activity and specificity. Thus pronase was selected for our degradation assay.[54] The peptides containing N-methylated peptide bonds at least at every second position, that is, peptides **2**–**4** and peptides **7**–**8**, were completely resistant towards pronase degradation for the whole assay period (24 h), see Table 2. It is evident from the corresponding HPLC-chromatogram (Supplementary Figs. S1–S8) that whenever the peptides contained N-methylated peptide bonds their chromatograms were quite reproducible even after pronase treatments; whereas when their non N-methylated analogues were incubated in the same way with pronase their chromatograms were drastically changed (Supplementary Figs. S1–S8).

Supplementary Figure S1 represents the ESI-MS of peptide **1** after pronase treatment for 24 h. There are two prominent peaks at m/z 695.0 and 548.0 which corresponds to peptides Ac-KLVFF-OH and Ac-KLVF-OH, generated after degradation of peptide **1** by pronase, along with a peak at m/z 765.1 for intact peptide **1**. Similarly, there is one prominent peak at m/z 343.8 in the ESI-MS of pronase treated peptide **5** which corresponds to the molecular mass of a scissored part of peptide **5** that is Ac-IGL-OH (in Supplementary Fig. S5).

Table 1
Aqueous solubility of peptides **1**–**8** as determined by the shake-flask solubility method, and measured-chromatographic lipophilicity (*Elog D*) as well as calculated (Clog *P* and ACD log *P*) lipophilicities

Peptide	Solubility (μM)	*Elog D*	Clog *P*[b]	ACD log *P*[c]
1	32	1.32	2.0	2.5
2	1583	2.14	3.2	3.0
3	>2500	2.10	3.8	2.5
4	899[a]	1.91	5.0	4.0
5	3	1.28	0.5	0.5
6	>2500	1.32	1.6	1.0
7	>2500	2.26	2.1	0.8
8	>2500	2.37	3.2	2.0

1: Non N-methylated peptides; **2**: two N-methylations on 3rd and 5th residues; **3**: three N-methylations on 2nd, 4th, and 6th residues; **4**: five N-methylations on 2nd, 3rd, 4th, 5th, and 6th residues; **5**: non N-methylated peptides; **6**: two N-methylations on 2nd and 6th residues; **7**: three N-methylations on 1st, 3rd, and 5th residues; **8**: five N-methylations on 1st, 2nd, 3rd, 5th, and 6th residues.
[a] Uncertain due to singlicate measurement on a small amount of material.
[b] Calculated Clog *P* values according to ChemDraw Ultra v. 11.0.
[c] Calculated using ACD Labs v. 12.0.

Table 2
Enzymatic degradation experiments with peptide **1**–**8**, as percentages of parent peptides remaining after 24 h of pronase treatment[a]

Peptide	% of parent remaining after Pronase degradation (24 h)
1	36
2	96
3	94
4	98
5	20
6	60
7	94
8	100

1: Non N-methylated peptides; **2**: two N-methylations on 3rd and 5th residues; **3**: three N-methylations on 2nd, 4th, and 6th residues; **4**: five N-methylations on 2nd, 3rd, 4th, 5th, and 6th residues; **5**: non N-methylated peptides; **6**: two N-methylations on 2nd and 6th residues; **7**: three N-methylations on 1st, 3rd, and 5th residues; **8**: five N-methylations on 1st, 2nd, 3rd, 5th, and 6th residues.
[a] Calculated from the area under the chromatograms recorded at 220 and 254 nm.

N-Methylated peptide **6** represents an interesting exception, as this peptide was degraded to some extent (40% after 24 h). This peptide contains two methylated peptide bonds that are separated by three intervening normal amide bonds. This unique positioning of N-methylated peptide bonds makes it more vulnerable to enzymatic degradation, as compared to the other N-methylated peptides (i.e., peptides **2**, **3**, **4**, **7**, and **8**). Notably, peptide **2**, which also contains only two N-methylated amide bonds, are stable suggesting that N-methylation at every second position is needed in order to render poly-N-methylated peptides completely resistant to enzymatic degradation.

2.4. Permeability assay

The permeability of the peptides across epithelial cells was studied using 2/4/A1 cells. Compared to Caco-2 cells, 2/4/A1 cells form a less tight monolayer, which is closer in comparison to the small intestinal epithelium and thus makes 2/4/A1 cells more suitable for the study of paracellular transported molecules.[55] Permeability experiments were performed both in presence and absence of a protease inhibitor cocktail. As suggested from the enzymatic degradation experiments above, there were no significant differences in compound recovery and permeability coefficient (P_{app}) in the presence or absence of the inhibitor cocktail, indicating that also the non-methylated peptides **1** and **5** were stable to protease degradation during the short transport experiments (see Table 3). In general, the compounds displayed a good mass balance with recoveries exceeding 80%.

The permeability of the peptides across the cell monolayer was slightly lower than that of the paracellular permeability marker mannitol ($P_{app} = 4.5 \pm 0.6 \times 10^{-6}$ cm s^{-1}) (see Table 3). For each series (peptides **1**–**4** and peptides **5**–**8**, respectively), a statistically significant linear relation between the degree of methylation and the apparent permeability coefficient was observed ($P = 0.02$ for peptides **1**–**4** and $P = 0.001$ for peptides **5**–**8**) (Fig. 2), that is, the permeability decreased with increasing degree of methylation. These results complement, and support, the observations made by Malakoutikah et al.,[45] who concluded that the permeability of poly-N-methylated peptides through artificial PAMPA membrane drastically decreased when the peptide chain became longer than four amino acid residues. It should be noted that despite the low apparent permeability of peptide **8** observed here, this peptide displayed marked in vivo efficacy in a *Drosophila melanogaster* model of Alzheimer's disease.[22]

2.5. Cytotoxicity assay

The effect of the two series of N-methylated hexapeptides on the viability of rat pheochromocytoma (PC 12) cells was studied by the MTT assay. The peptides were added to the cells in six different concentrations (1, 5, 10, 50, 100, and 200 μM) and incubated for 4 h at 37 °C. The peptides were found to be non-toxic to the cells (Fig. 3). The viability of the peptides **1**–**8** were 95% and above under mentioned experimental conditions (up to 200 μM). These results demonstrate that poly-N-methylated peptides do not show

Table 3
Apparent permeability coefficients (P_{app}) for the transepithelial transport of peptides **1**–**8** across 2/4/A1-monolayers (6 days after seeding); measurements done in buffered HBSS pH 7.4 ($n = 3$)

Peptide	P_{app} [$\times 10^{-6}$ cm s^{-1}] in absence of protease inhibitors	P_{app} [$\times 10^{-6}$ cm s^{-1}] in presence of protease inhibitors
1	2.8 ± 0.7	3.4 ± 0.5
2	n.d.	1.7 ± 0.3
3	n.d.	2.5 ± 0.2
4	2.3 ± 0.4	1.9 ± 0.2
5	3.1 ± 0.6	3.4 ± 0.1
6	2.2 ± 0.2	n.d.[a]
7	1.6 ± 0.2	n.d.[a]
8	1.4 ± 0.5	1.8 ± 0.2

1: Non N-methylated peptides; **2**: two N-methylations on 3rd and 5th residues; **3**: three N-methylations on 2nd, 4th, and 6th residues; **4**: five N-methylations on 2nd, 3rd, 4th, 5th, and 6th residues; **5**: non N-methylated peptides; **6**: two N-methylations on 2nd and 6th residues; **7**: three N-methylations on 1st, 3rd, and 5th residues; **8**: five N-methylations on 1st, 2nd, 3rd, 5th, and 6th residues.
[a] Not done.

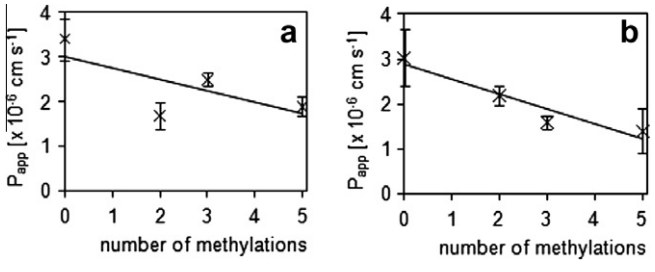

Figure 2. Observed permeability across a monolayer of 2/4/A1 cells as a function of the number of *N*-methyl amide bond for (a) peptides **1**–**4** and (b) peptides **5**–**8**.

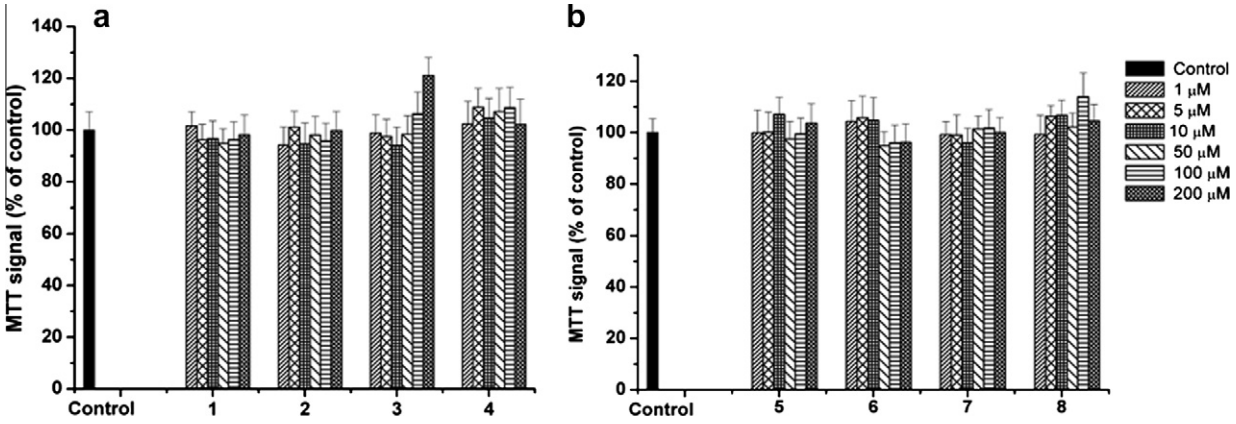

Figure 3. Cell toxicity to PC12 cells. Cellular response of PC12 cells treated with peptides for 4 h. (a) Peptides **1**–**4** and (b) peptides **5**–**8**. Control treatment is set to 100%. Error bars represent standard error of the mean ($n = 6$).

toxicity in the MTT assay, or in the 2/4/A1 cells used in the permeability experiments above, suggesting that these peptides lack general toxicity.

3. Conclusion

In this report we have shown that poly-N-methylated peptides are stable towards proteolytic degradation when N-methylation is introduced at least at every second amino acid residue as previously showed in their series of anti-amyloids based on the 16–22 portion of Aβ-peptide by Gordon et al.[43] Poly-N-methylated peptides were also shown to lack general cytotoxicity, as demonstrated by measuring the cell viability of PC12 cells. In addition, it was demonstrated that N-methylation is a powerful methodology for increasing the aqueous solubility of peptides. A permeability assay, using an epithelial cell monolayer, verified the high proteolytic stability of the poly-N-methylated peptides, but also ascertained that the permeability decreased upon incorporation of an increasing number of N-methylated amide bonds. Although these data suggests that a lower degree of N-methylation and/or use of short peptide sequences (<6 residues) is advantageous in order to have good oral bioavailability of such substances, it should be kept in mind that we previously showed in vivo efficacy of poly-N-methylated peptide **8** in a transgenic Drosophila model which both has an intestinal system, and a CNS barrier similar to the BBB found in higher organisms, and that Adessi et al. showed that the peptide with the sequence Ac-LP-(NMe)-FFD-NH_2 was able to pass the BBB in mouse after an iv infusion, most likely through an active transport. In this study we utilized eight hexapeptides, which correspond to N-methylated analogues of residues 16–21 and 32–37 of the Aβ-peptide known to inhibit aggregation of Aβ fibrillation in vitro. We envisage that the conclusions obtained herein are representative for poly-N-methylated peptides in general, and hope that the results will stimulate further development of poly-N-methylated peptides as biologically active tools and as lead compounds.

4. Experimental

4.1. Peptides

Peptides **1**–**8** (Fig. 1) were synthesized using solid-phase peptide synthesis, as described elsewhere.[23–28] All the experiments were carried out with purified, lyophilized peptides.

4.2. Solubility

Solid material in excess in 1 ml 0.1 M phosphate buffer, pH 7.4, was incubated at 22 °C (ambient room temperature) for 24 h on an orbital shaker (300 rpm). Undissolved material in the peptides (run in duplicate) was then centrifuged down twice (3000 rpm, °C 22, 15 min) and the resulting supernatant was analyzed by Waters AB Aquity UPLC-UV/MS system. Separation was performed on a reverse phase Aquity UPLC BEH C18 (2.1 × 50 mm, 1.7 μm) column using a mobile phase system consisting of 10 mM ammonium acetate containing 5–95% acetonitrile in a LC-gradient of 4.5 min. MS-identification was performed in ESI-MS positive and negative mode. Quantification of the solubility was performed against a UV calibration curve in the range 1–400 μM with an upper limit for quantification of 2.5 mM.

4.3. Lipophilicity

The chromatographic *E*log *D*-value[53] was determined by comparing the retention time of a compound with unknown log *D*, with a linear calibration curve based on the retention time of five different standards with known log *D*-values; Metoprolol, Warfarin, Propranolol, Testosterone, and Felodipine. The calibration solution is prepared from 10 mM stock solutions of the standard compounds in DMSO. The stock solutions were pooled and diluted with DMSO to a final concentration of 100 μM. For analysis 2 μl of the peptide stock solution (10 mM) was diluted with 200 μL of DMSO. Analysis was performed with a Waters AB Aquity UPLC-UV/MS system. Separation was performed on a reverse phase Aquity UPLC BEH C18 (2.1 × 50 mm, 1.7 >μm) column using a mobile phase system consisting of 10 mM ammonium acetate containing 5–95% acetonitrile in a LC-gradient of 4.5 min. The estimated *E*log *D* values were evaluated from the monoisotopic ions in ESI+ providing a MS-confirmation of identity as well. To monitor the robustness of the analysis over time the calibration solution was analyzed at the beginning and at the end of each batch of samples. In addition, a quality control (QC) sample was analyzed at the beginning and at the end of each batch of 12 samples (every peptide was run in triplicate). The QC-compound was cyclobenzaprine (Monoisotopic formula weight 275.2). In order for an analyzed dataset to be approved, the obtained value of the QC-compound should be within two standard deviations of the calculated average value.

4.4. Stability towards enzyme degradation

For each degradation experiment stock solutions of the peptidic substrates (peptides **1**–**8**) were made using 20 mM Tris buffer, pH 8.9 at a peptide concentration of 1 mM. The enzyme stock solution of pronase (EC 3.4.24.4; protease from Streptomyces griseus, Fluka, ~6 Unit/mg) was prepared in a solution of phosphate buffered saline (PBS) pH 7.4 at a concentration of 0.1 μM. A 100 μl solution of

each substrate (peptides **1**–**8**) in a Tris buffer (pH 8.9) and a 0.5 μl solution of enzyme solution were mixed and incubated at 37 °C for 24 h. Following incubation, 200 μl of cold acetonitrile was added to precipitate the enzyme and the solutions were centrifuged for 5 min at a 10,000*g*. The resulting supernatants were analyzed by HPLC–MS; Gilson System consisting of a Gilson 322 pump, Gilson 234 auto-injector and a Gilson UV/VIS 152 detector coupled in tandem with a Finnigan AQA mass spectrometer and an ELSD (SEDEX-LT) from Sedere. The reverse phase HPLC analyses were performed using a phenomenex Gemini C18-column (5 μm; 150 × 3.00 mm). For all the samples ESI-MS were performed in positive mode.

4.5. Permeability assay

The studies were performed on 2/4/A1 cell monolayers (passage 34–36) grown on cell culture inserts. In short, 2/4/A1 cells were expanded at 33 °C in RPMI1640 medium supplemented with 4% fetal bovine serum (v/v) (Gibco, Invitrogen AB, Lidingo, Sweden), 2 mM L-glutamine, 1 mg/ml BSA, 65 ng/ml dexamethasone, 20 ng/ml epidermal growth factor, 20 ng/ml murine epidermal growth factor, ITS premix containing 10 μg/ml insulin, 5.5 μg/ml transferring, and 5 ng/ml selenic acid according to previously published procedures.[55] For transport studies, 2/4/A1 cells were seeded on polycarbonate cell culture inserts (0.18×106 cells/cm^2, Transwell® system, diameter 12 mm, pore size 0.4 μm, Corning Costar, The Netherlands) coated with ECM gel from Engelbreth–Holm–Swarm murine sarcoma (16 μg/cm^2) and were allowed to differentiate at 39 °C (5% carbon dioxide) for 5 days in Optimem (Gibco, Invitrogen AB, Lidingo, Sweden) supplemented with 2 mM L-glutamine, 1 mg/ml BSA, 65 ng/ml dexamethasone, ITS premix containing 10 μg/ml insulin, 5.5 μg/ml transferring, and 5 ng/ml selenic acid, 1 nM T3 (triiodo-L-thyronine), 2 mM Na-pyruvate, 1% 100× penicillin streptomycin and 0.1% DMSO. The transepithelial resistance values of the monolayers were 51 ± 5 Ω cm^2 (n = 48 filters) before the experiment and had decreased in average 5 Ω cm^2 after the transport studies.

Briefly, transport experiments were performed in Hank's balanced salt solution (HBSS) buffered with 25 mM Hepes at pH 7.4 and in presence of 0.5% DMSO on the apical side of the monolayer (from the peptide stock solution). 50 μM of the peptide solution was added apically at time 0 and samples were withdrawn after 10, 20, and 30 min (the volume was replaced by pre-tempered HBSS). The quantification was done by HPLC–MS/MS (ThermoFinnigan TSQ Quantum Discovery triple–quadrupole (ESI) coupled to a Surveyor autosampler and Surveyor HPLC–MS-pump a XTerra C18-column (2.1 × 100 mm, Waters) was used for chromatography).

The apparent permeability coefficients (P_{app}) were calculated according to $P_{app} = dQ/dt \times 1/(A \times C_0)$, where dQ/dt is the steady-state flux (μmol/s), A is the surface area of the filter (cm^2) and C_0 is the initial concentration in the donor chamber at each time interval (μM).

For experiments in presence of protease inhibitors, a cocktail of 1 mM diprotin A (Bachem), 1 mM captopril (Fluka) and 0.29 mM bestatin HCl (Bachem) was added during the experiment.

Toxicity of the peptides against the cell monolayers was tested by tracing the [^{14}C]-mannitol-permeability in absence and in presence of peptide **1** and peptide **8**, respectively; there was no statistically significant effect of the peptides on mannitol-permeability, that is, there was no sign for toxicity of the peptides against the 2/4/A1-monolayers.

A linear regression of permeability versus methylation degree was performed and a *t*-test of the slope factor was applied to test the statistical significance of the correlation between methylation degree and permeability. Minitab was used in the regression and significance tests.

4.6. Cytotoxicity assay

Rat pheochromocytoma (PC 12) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 10% horse serum and penicillin/streptomycin in humidified atmosphere of 5% CO_2 at 37 °C. Cells were sub-cultured and grown in collagen-coated tissue culture flasks and were used for the experiments when they were 70% confluent. Cytotoxicity of the peptides was determined by measuring the cells' ability to reduce MTT to MTT formazan which reflects viability of the cells. Cells (1×10^4 cells/well) in their exponential growth phase were seeded into each well of a 96-well culture plate (Sarstedt, Sweden) and incubated overnight in a CO_2 incubator. The following day the media was exchanged with 45 μl/well of DMEM without phenol red supplemented with 10% FCS and penicillin/streptomycin. Thereafter, peptides (at final concentration of 1, 5, 10, 50, 100, and 200 μM) were added (5 μl/well) and incubated for 4 h in a CO_2 incubator. 50 μl MTT (at final concentration 0.3 mg/ml in DMEM without phenol red) was then added to each well and incubated for another 2 h. The cells were then lysed with 100 μl of lysis buffer (50% DMF and 20% SDS in water) overnight at 37 °C. Absorbances at 592 nm were measured using a microplate reader. Cells without the treatments served as positive control and their assay values were taken as 100%. All readings were carried out in triplicate and the experiment was repeated thrice to check the reproducibility of the data. The peptides **1**–**8** were initially dissolved in DMSO at 5 mM concentration and finally diluted to above-mentioned concentration in 10 mM phosphate buffer pH 7.4.

Acknowledgments

This work was supported by the Foundation Olle Engkvist Byggmästare, Vetenskapsrådet (The Swedish Research Council), and a Sweden–South African Research Link Grant. The Wenner-Gren foundation is gratefully acknowledged for a post doctoral fellowship to TG (2005–2006), and Johan Hammarbäck, AstraZeneca, for help with solubility measurements.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.087.

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