Development of novel small-size peptides as putative therapeutic drugs
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Neuroprotective action & neutralization of Aβ₄₂-induced memory impairment by a novel in silico designed N-methyl amino acids containing peptide


Abstract

We report the in silico design and the neuroprotective properties of Aβ₄₂ derived synthetic N-methyl amino acids containing penta-peptide Ac-Lys-(Me)Ile-Ile-(Me)Gly-Leu-NH₂. These data demonstrate the ability of this novel peptide to interact with different regions of Aβ₄₂ which may be targeted selectively. Furthermore, this study provides proof of principle that this rationally designed peptide has protective properties against the neurotoxic effects of oligomeric Aβ₄₂ in vitro and in vivo. This peptide can serve as a crucial molecular model design for development of therapeutic molecules for treatment of Alzheimer’s disease.
7.1 Introduction

Alzheimer’s disease (AD) is a complex multifactorial neurodegenerative syndrome characterized by the patient’s memory loss and impairment of a wide range of cognitive abilities. This devastating disease affects more than 20 million people worldwide and, as a consequence of the world’s aging population, the prevalence of AD is expected to increase to endemic dimensions both in developed and developing nations (Blennow et al. 2006; Pratico and Delanty 2000). AD is the most extensively studied amyloid-based disease, whose main hallmarks are characterized by pathological high levels of amyloid deposits in frontal brain regions (senile or amyloid or neurotic plaques) and neurofibrillary tangles (Haass and Selkoe 2007; Holtzman and Mobley 1991). The major components of senile plaques are small peptides of 39-43 amino acids called β-amyloid (Aβ) which are produced through endoproteolysis of the amyloid precursor protein (APP). During the pathogenesis of AD the equilibrium of Aβ production and Aβ clearance is disturbed, which eventually leads to elevated Aβ levels, increased Aβ aggregation and consequently impaired memory function (Wasling et al. 2009). Recent evidence now indicates that particularly soluble oligomeric forms of Aβ are to be considered as the most toxic form of this peptide, and correlate well with the progression and cognitive decline of the disease (Lesne et al. 2006; Wasling et al. 2009).

Due to the high incidence that AD has in the human aging population, there is an evident need of therapeutic agents that could at least significantly delay the course of the disease (Hardy and Selkoe 2002; Wolfe 2002). Several research strategies currently attempt to develop therapies and therapeutic agents that aim to reduce Aβ production, enhancing its clearance and/or preventing or retarding the amyloidogenic processes. Amongst them, the use of peptides or peptidomimetic molecules derived from the Aβ sequence is particularly appealing (Granic et al. 2009; Soto et al. 1998; Tjernberg et al. 1996; Wolfe 2002). Promising putative treatments may be those designed to inhibit steps that precede Aβ peptide aggregation, by blocking the production of toxic soluble Aβ oligomers in the first place, or by reversing, somehow, the toxic effect of these soluble oligomers (Figure 7.1).

In fact, some short peptides that are derived from the Aβ sequence have already been reported to specifically interact with Aβ and cause interferences with its neurotoxic effects. LPFFD (Soto et al. 1996; Soto et al. 1998), KLVFF (Hetenyi et al. 2002b; Tjernberg et al. 1997; Tjernberg et al. 1996), RIIGLa (Fulop et al. 2004) and LPYFDa (Datki et al. 2003; Datki et al. 2004; Granic et al. 2009; Juhasz et al. 2009; Szegedi et al. 2005) are some of the promising starting points to develop potential drugs that can somehow reverse the negative impacts of soluble Aβ aggregates, although their underlying molecular mechanisms remain partly elusive. In order to increase the anti-amyloidogenic properties of these peptides, a new strategy was recently explored with the introduction of N-methyl amino acids in these sequences (Cruz et al. 2004; Gordon et al. 2001; Gordon et al. 2002). N-Methyl amino acids have been used in several systems to control, or prevent the aggregation of β-sheet and β-strand peptides (Chitnumsub et al. 1999; Clark et al. 1998; Doig 1997; Hughes et al. 2000; Nesloney and Kelly 1996; Rajarathnam et al. 1994). The goal of this modification is to block the hydrogen bond network that stabilizes the β-sheet amyloid structure and this way to inhibit the formation of toxic oligomers and/or amyloid aggregates.

In the present study, we report the in silico design of a new Aβ-derived synthetic penta-peptide Ac-Lys-Ile-Ile-Gly-Leu-NH₂ (PN20) and its chemically modified version; Ac-Lys-(Me)Ile-Ile-(Me)Gly-Leu-NH₂ (PN22), which contains alternated N-methyl amino acids (Scheme 7.1). The protective property of these
The rational in silico design of PN20 and PN22

In this study we rationally designed two novel aggregation modulating peptides (PN20 and PN22). Their in silico design was based on the conformational behavior of Aβ₄₂ aggregates, which was previously reported by Masman and coworkers (Masman et al. 2009a). The portion β₂ of these aggregates, specifically the highly hydrophobic sequence Ala₃₀-Ile₃₁-Ile₃₂-Gly₃₃-Leu₃₄, was the starting point of the present design. These peptides were designed based on the observations of the conformational behavior of the Aβ₄₂ aggregates in silico previously reported by Masman and coworkers (Masman et al. 2009a). The portion β₂ of these aggregates, specifically the highly hydrophobic sequence Ala₃₀-Ile₃₁-Ile₃₂-Gly₃₃-Leu₃₄, was the starting point of the present design.
by aminopeptidases is enhanced; and (iv) peptide ends are blocked against synthetase activities. Moreover, C-terminal amidation is essential to the biological activity of many neuropeptides and hormones (Fricker 2005; Kim and Seong 2001). These two modifications were our guiding principle to the design of PN20.

In fact, a possibility to counteract the detrimental effects of oligomeric Aβ is by modulating its aggregation process. A very appealing strategy to endow peptides with aggregation modulating properties is the incorporation of non-natural amino acids to their sequence. Such is the case of N-methyl amino acids containing peptides. When the N-methyl amino acids residues are located in an alternated fashion into the peptide sequence, and the peptide has the possibility to form extended conformations, e.g. β-sheets, two different “faces” are expressed. On the one hand, one of the faces has all the normal H-bonding possibilities that a natural peptide has (the so-called “rich H-bonding face”). On the other hand, the other face is limited on H-bonding possibilities due to the presence of the N-methyl groups (Scheme 7.1). Thus, the incorporation of N-methyl-L-isoleusine and N-methyl-glycine (or sarcosine) in position 2 and 4 respectively, by replacement of their natural analogs, led us to PN22.

### 7.2 Material and methods

#### 7.2.1 Molecular modeling

##### 7.2.1.1 Stochastic conformational search. EDMC calculations

The conformational space of the pentapeptides PN20 and PN22 was explored using the method previously employed by Liwo et al. (Liwo et al. 1996a) which includes the electrostatically driven Monte Carlo (EDMC) method (Ripoll and Scheraga 1988, 1990) implemented in the ECEPP/3 package. Conformational energy was evaluated using the ECEPP/3 force field (Némethy et al. 1992). Hydration energy was evaluated using a hydration-shell model with a solvent sphere radius of 1.4 Å and atomic hydration parameters that have been optimized using nonpeptide data.
In order to explore the conformational space extensively, we carried out 20 different runs, each of them with a different random number. Therefore, we collected a total of 5000 accepted conformations. Each EDMC run was terminated after 250 energy-minimized conformations had been accepted. The parameters controlling the runs were the following: a temperature of 298.15 K was chosen for the simulations. A temperature jump of 50,000 K was used; the maximum number of allowed repetitions of the same minimum was 50. The maximum number of electrostatically predicted conformations per iteration was 400; the maximum number of random-generated conformations per iteration was 100; the fraction of random/electrostatically predicted conformations was 0.30. The maximum number of steps at one increased temperature was 20; and the maximum number of rejected conformations until a temperature jump was executed was 100. Only trans peptide bonds ($\omega \approx 180^\circ$) were considered. All accepted conformations were then clustered into families using the program ANALYZE (Meadows et al. 1994a; Pohorille and Pratt 1990) by applying the minimal-tree clustering algorithm for separation, using all heavy atoms, energy threshold of 30 kcal mol$^{-1}$, and RMSD of 0.75 Å as separation criteria. This clustering step allows a substantial reduction of the number of conformations and the elimination of repetitions. A more detailed description of the procedure used here is given in section 4.4 Computational methods of (Masman et al. 2009b).

### 7.2.1.2 Molecular dynamics simulations

The most populated conformation of PN20 and PN22 obtained from the EDMC were embedded in a cubic box containing SPC waters, (Berendsen et al. 1981b) leaving at least 10 Å between the solutes and the edge of the box. The total number of water molecules varied between 15630 and 25919. Cl ions were added to the systems by replacing water molecules in random positions, thus making the whole system neutral. Multiple simulations were performed for each system, starting from different initial random velocity distributions. Details of the equilibration procedure can be found in the Suppl. Information. For each system 10 ns production runs were obtained and analyzed. The coordinates were saved every 2 ps. Molecular dynamics (MD) simulations were performed using the GROMACS 3.3.2 package of programs (Van Der Spoel et al. 2005), with the OPLS-AA force field (Kaminski et al. 2001). The simulations were run under NPT conditions, using Berendsen’s coupling algorithm (Berendsen et al. 1984) for keeping the temperature and pressure constant ($P = 1$ bar, $\tau_P = 0.5$ ps; $T = 310$ K, $\tau_T = 0.1$ ps). The LINCS algorithm (Hess et al. 1997) was used to constrain the lengths of hydrogen containing bonds; the waters were restrained using the SETTLE algorithm (Miyamoto and Kollman 1992). The time step for the simulations was 0.002 ps and the compressibility $4.8 \times 10^{-5}$ bar$^{-1}$. Van der Waals forces were treated using a 1.2 nm cutoff. Long-range electrostatic forces were treated using the particle mesh Ewald method (PME) (Darden et al. 1993a). The analysis of the simulations was performed using the analysis tools provided in the Gromacs package. The root mean square deviation (RMSD) of backbone atoms, the total and potential energies were calculated. The root mean square fluctuation (RMSF) of the backbone atoms and the hydrophilic, hydrophobic and total Solvent Accessible Surface Area (SASA) were also determined. The total number of hydrogen bonds in the peptide group were quantified by counting acceptor and donor atom pairs that are not further apart than 0.35 nm. All molecular graphical presentations were created by VMD (Humphrey et al. 1996) and/or UCSF Chimera (Pettersen et al. 2004a) packages. The standard deviation of every given value is shown in between brackets.

### 7.2.1.3 Docking studies

Two models for $\alpha\beta$ were used as target systems; the monomeric $\alpha\beta_{42}$ elucidated by
Crescenzi et al (monomeric model) (Crescenzi et al. 2002), PDB code 1IYT, and the pentameric aggregate Aβ$_{42}$ developed by Masman et al (pentameric model) (Masman et al. 2009a). The structures were prepared for docking study as follows: for the Aβ$_{42}$ molecules, water molecules were removed from the PDB file and hydrogen atoms were added; Gasteiger charges, atomic solvation parameters and fragmental volumes were merged to the target system. For all peptides, the structure of the most populated family (results from the EDMC calculations) was taken as initial conformation. Gasteiger charges were assigned and non-polar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The docking energy grid was produced with the auxiliary program AutoGrid. The grid dimensions were 61x61x61 for the monomeric model, and 90x60x60 for the pentameric model, points along the x-, y- and z-axes, with points separated by 0.375 Å. The grids were chosen to be sufficiently large to cover significant portions of the putative binding sites. The center of the pentapeptide was positioned at the grid center. All graphic manipulations and visualizations were performed by means of the AutoDock Tools (Sanner 1999) and the Chimera (Pettersen et al. 2004a) programs, and ligand docking with AUTODOCK 4 (Morris et al. 2009). The Lamarckian genetic algorithm was utilized and the energy evaluations were set at 2.5x10$^6$. A total of 250 accepted conformations were collected. Other parameters were set to default values.

7.2.2 Experimental section

7.2.2.1 Compounds

The peptides Ac-Lys-Ile-Ile-Gly-Leu-NH$_2$ (PN20), Pro-Aβp-Tyr-Leu-Phe-NH$_2$ (scrP) and Amyloid β peptide 1−42 (Aβ$_{42}$) were purchased from EZBiolab Inc.(Carmel, USA). Anti-Aβ antibody (6E10) was obtained from Covance (Emeryville, USA). The peptide Ac-Lys-(Me)Ile-Ile-(Me)Gly-Leu-NH$_2$ (PN22) was purchased from AnaSpec Inc (San Jose, CA, USA). HPLC purity higher than 95% was described for all peptides used here. Other compounds used in this study were purchased from Invitrogen (Carlsbad, USA) or Sigma-Aldrich Corporation (St. Louis, USA).

7.2.2.2 Preparation of Aβ$_{42}$-oligomers

Oligomeric Aβ$_{42}$ was prepared as described by Granic and colleagues (Granic et al. 2009). Also, the aggregational state and the secondary structure of the oligomeric Aβ$_{42}$ preparation were examined by sodium dodecyl sulfate (SDS)-PAGE Western blotting and Circular Dichroism (CD) spectrometry. For a more detailed description of the methodology used here, see Granic et al. (2009).

7.2.3 In vitro testing

7.2.3.1 Primary cortical neuron culture

Primary cortical neurons were prepared from embryonic brains (E14) of C57Bl/6J mice. The cortices were carefully dissected, meninges were removed and the neurons separated by trituration. Cells were plated on poly-D-lysine pre-coated plates at a density of 1.2 x 10$^5$ cells/well (96 well plates). Neurobasal medium supplemented with 2% (v/v) B27-supplement, 0.5 mM glutamine and 1% (v/v) penicillin/streptomycin was used as a culture medium. After 48 h neurons were treated with 10 μM cytosine arabinoside for another 48 h to inhibit non-neuronal cell growth. Subsequently, the medium was completely exchanged and after 6 days of in vitro culture, the neurons were used for experiments.

7.2.3.2 Treatment of cells

Possible toxicity of the penta-peptides used here and Aβ$_{42}$ oligomers was determined by incubating neuronal cultures for 24 h with different concentrations of the peptide solutions. The neuroprotective effect of PN20 and PN22 was assessed by incubating neurons (cultured in 96 well plates) for 24 h with 25 µM oligomeric Aβ in the presence or absence of different concentrations of
PN20 or PN22 peptides. After treatments, the medium was completely exchanged, and 24 h later, the cell viability determined by an MTT-assay. All treatments were performed in triplicates and the experiments were repeated at least two times.

7.2.3.3 **Determinant of Cell Viability by MTT-Assay**

Neuronal viability was determined by the colorimetric MTT \([3-(4,5\text{-dimethylthiazol-2-yl})-2,5\text{-diphenyltetrazolium bromide}]\) assay as described previously (Mosmann 1983). 1.25 mg/ml MTT solution was added to each well of a 96 well plate. After 2 h of incubation, cells were lysed in acidic propan-2-ol solution (37% HCl/ propan-2-ol: 1/166). The absorbance of each well was measured with an automated ELISA plate reader (Bio-Rad, Munich, Germany) at 595 nm with a reference filter at 630 nm.

7.2.4 **In Vivo Testing**

7.2.4.1 **Animals**

Behavioral experiments were performed with 9–12 weeks old male C57Bl/6J mice (Harlan, Horst, The Netherlands). Individually housed mice were maintained on a 12 h light/dark cycle (lights on at 7.00 a.m.) with food (Hopefarms, standard rodent pellets) and water ad libitum. A layer of sawdust served as bedding. The animals were allowed to adapt to the housing conditions for 1–2 weeks before the experiments started. The procedures concerning animal care and treatment were in accordance with the regulations of the Ethical Committee for the use of experimental animals of the University of Groningen (License number DEC.4668D).

7.2.4.2 **Animal Surgery**

Double guide cannulae type C235 (Plastics One, Roanoke, USA) were implanted in the brain using a Kopf stereotactic instrument during Hypnorm/Midazolam (10 ml/kg, i.p.) anesthesia under aseptic conditions as previously described (Nijholt et al. 2008) with anteroposterior (AP) coordinates zeroed at Bregma directed toward both dorsal hippocampi (i.h.), AP -1.5 mm, lateral 1 mm, depth 2 mm (Franklin and Paxinos 1997). Each double guide cannula with inserted dummy cannula and dust cap was fixed to the skull with dental cement (3M ESPE AG, Seefeld, Germany). Administration of 1 mg/ml finadyne (2.5 mg/kg s.c.) before the surgery served as analgesic. The animals were allowed to recover for 6–7 days before the behavioral test.

7.2.4.3 **Intrahippocampal Injections**

Bilateral i.h. injections were performed under short isoflurane anesthesia using a Hamilton microsyringe fitted to a syringe pump unit (TSE systems, Bad Homburg, Germany) at a constant rate of 0.3 µl/min (final volume: 0.3 µl per side). Oligomerized Aβ\(_{42}\) was injected in a final concentration of 30 pmol and PN22 or scrP in a final concentration of 150 pmol into the dorsal hippocampus. PBS (pH 7.5) served as vehicle. ScrP was used as a sequence control peptide. One hour after the injection the animals were subjected to a training session in a fear conditioning paradigm (scheme 7.2). The number of animals per group varied from 7 to 10.

7.2.4.4 **Fear Conditioning**

Fear conditioning was performed in a plexiglas cage (44x22x44 cm) with constant illumination (12 V, 10W halogen lamp, 100–500 lux). The training (conditioning) consisted of a single trial. Before each individual mouse entered the box, the box was cleaned with 70% ethanol. The
mouse was exposed to the conditioning context for 180 s followed by a scrambled footshock (0.7 mA, 2 s, constant current) delivered through a stainless steel grid floor. The mouse was removed from the fear conditioning box 30 s after shock termination to avoid an aversive association with the handling procedure. Memory tests were performed 24 h after fear conditioning. Contextual memory was tested in the fear conditioning box for 180 s without footshock presentation. Freezing, defined as the lack of movement except for respiration and heart beat, was assessed as the behavioral parameter of the defensive reaction of mice by a time-sampling procedure every 10 s throughout memory tests. In addition, mean activity of the animal during the training and retention test was measured with the Ethovision system (Noldus, Wageningen, The Netherlands). Immediately after the behavioral test mice were injected i.h. with methylene blue solution during sodium-pentobarbital anesthesia (0.1 ml/10g, i.p.). Brains were removed and serially sectioned at 50 μm. Sections were stained on glass for 5 min in 0.1% nuclear fast red solution. To identify the location of the injection, sections were analyzed using light microscopy. Only data from animals in which the proper intrahippocampal site of injection was confirmed, were evaluated.

7.2.5 Statistical analysis

Behavioral data were analyzed by analysis of variance (ANOVA) followed by the Bonferroni post-hoc test to determine statistical significance. For statistical analysis of the MTT assays, an unpaired Student’s t test with unequal variance was used. A p-value *< 0.05 was considered to be statistically significant. A p-value **< 0.005 was considered to be highly significant. Data are presented as mean value ± standard error of the mean (SEM).

Results

7.3 Molecular modeling

7.3.1 Stochastic conformational search in solution. EDMC calculations

In order to have a better view at the molecular level, it is crucial to assess the conformational behavior of PN20 and PN22 in solution. Therefore, all peptides were selected for energy calculations to determine the relevant conformations. The results of the theoretical calculations are summarized in Table 7.1 in Appendix A. Calculations yielded a large set of conformational families for each peptide studied. The total number of conformations generated was 70236 and 79077, for PN20 and PN22 respectively, whereof 5000 conformations for each pentapeptide were accepted. In the clustering procedure, an R.M.S.D (Root Mean Square Deviation) of 0.75 Å and a ΔE of 30 kcal mol⁻¹ were used. The number of families after clustering was 703 and 409, for PN20 and PN22, respectively. The total number of families accepted with a relative population higher than

<table>
<thead>
<tr>
<th>Lys</th>
<th>Ile/(Me)Ile</th>
<th>Ile</th>
<th>Gly/(Me)Gly</th>
<th>Leu</th>
<th>d CαLys/CαLeu</th>
</tr>
</thead>
<tbody>
<tr>
<td>θ₁</td>
<td>Ψ₁</td>
<td>θ₂</td>
<td>Ψ₂</td>
<td>θ₃</td>
<td>Ψ₃</td>
</tr>
<tr>
<td>PN20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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<td>-26.75</td>
<td>-77.76</td>
<td>-36.70</td>
<td>-76.80</td>
</tr>
<tr>
<td>3</td>
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<td>-24.36</td>
<td>-79.41</td>
<td>-32.43</td>
<td>-76.68</td>
</tr>
<tr>
<td>PN22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-150.42</td>
<td>75.40</td>
<td>-120.16</td>
<td>79.15</td>
<td>-140.85</td>
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<td>76.58</td>
<td>-116.92</td>
<td>81.14</td>
<td>-55.16</td>
</tr>
</tbody>
</table>
0.50% was 30 and 11, for PN20 and PN22, respectively, that sum up to ca 85% of all conformations. All low-energy conformers of pentapeptides studied here were then compared to each other. The comparison involved the spatial arrangements, relative energy and populations. The PN20 evaluation showed that the most populated family (24.20%) is also the energetically preferred one, while its second most populated family (5.52%) has a relative energy of 2.55 kcal mol$^{-1}$ above the global minimum. The third most populated family (3.76%) shows a relative energy of 1.66 kcal mol$^{-1}$ above the global minimum. On the other hand, the most populated family of PN22 (62.38%) is also the global minimum, while its second most populated family showed a relative energy of 0.57 kcal mol$^{-1}$ above the global minimum and a relative population of 8.50%. The third most populated family (5.20%) has a relative energy of 0.61 kcal mol$^{-1}$ above the global minimum. It was observed that PN20 possesses a marked tendency to form folded conformations while PN22 shows high preference to form semi-extended or fully-extended conformations.

Table 7.1 shows the values of $\phi$ and $\psi$ torsional angles for the three most populated families of PN20 and PN22. Spatial overlapped stereoviews of selected conformations, for PN20 and PN22 are shown in Figure 7.2.

### 7.3.1.2 Molecular dynamics

PN20 and PN22 were examined by MD due to the need of exploring their conformational behavior in a more realistic system by means of an explicit solvent. Figure 7.3 shows the distance between the C$^a$ atoms of residues Lys$^i$ and Leu$^j$ ($d$ C$^a_{\text{Lys}}$ / C$^a_{\text{Leu}}$) as a function of the simulation time for PN20 and PN22. This parameter is a clear and easy way of depicting the conformational preferences of these peptides. In this figure, it is observed that PN20 prefers folded conformation with a $d$ C$^a_{\text{Lys}}$ / C$^a_{\text{Leu}}$ = 4.22(0.49) Å while PN22 showed a marked preference to extended conformations ($d$ C$^a_{\text{Lys}}$ / C$^a_{\text{Leu}}$ = 12.25(1.08) Å).

Figure 7.2: Stereoview of the three most populated families for PN20 and PN22 optimized at EDMC/SRFOPT/ECCEP/3 level of theory. (A) Overlapped geometries of family 1 ($\Delta E = 0.00$ cal mol$^{-1}$, white), family 2 ($\Delta E = 2.45$ kcal mol$^{-1}$, magenta) and family 3 ($\Delta E = 1.66$ kcal mol$^{-1}$, cyan) for PN20. (B) Overlapped geometries of family 1 ($\Delta E = 0.00$ kcal mol$^{-1}$, white), family 2 ($\Delta E = 0.57$ kcal mol$^{-1}$, magenta) and family 3 ($\Delta E = 0.61$ kcal mol$^{-1}$, cyan) for PN22. All hydrogen atoms and side-chains have been deleted for more clarity. N-Methyl groups are depicted in ball-and-stick model.

Figure 7.3: Distance between C$^a$ atoms of residues Lys$^i$ and Leu$^j$ as a function of simulation time. The maximum ideal (for a fully extended conformation, where all $\phi$ and $\psi \approx 180^\circ$) distance between the above mentioned atoms is also shown.
Chapter 7

7.3.1.3 Docking studies

The docking studies performed here have been analyzed on the basis of their conformational population distribution as a function of the binding energy. The clusterization procedure was run with a cut-off of 2.00 Å RMSD. Figure 7.4 shows the density population distribution for PN20 and PN22 on the two target system-models used here.

Monomeric Model. Two potential binding sites were found by using a single blind docking run (results not shown) on the monomeric Aβ₄₂ molecule (PDB code 1IYT). Figure 7.5A depicts the location of these potential binding sites. Site I encompasses the residues 21-26 containing Glu²² and Asp²³, which were previously identified as residues for aggregation of the oligomers (Buchete and Hummer 2007; Masman et al. 2009a). Site II includes residues 6-12 located in the portion of the molecule that loses all structural organization after oligomerization, thus forming part of the so-called disordered region. The docked-conformational population for PN20 and PN22 is shown in Figure 7.4, panel A. It can be observed that PN20 does not show a preference to bind either Site I or Site II. PN20 obviously binds both sites unselectively and with more or less the same intensity. On the other hand, the most populated conformations of PN22 show a binding preference for Site I. Thus, the binding of

Table 7.2: The two most populated families of PN20 and PN22 found by docking simulations on the monomeric and pentameric Aβ₄₂ peptide and the corresponding binding energy (EB, kcal mol⁻¹) of the complexes. The binding constant (KB, M⁻¹) and the relative populations (%P) are also shown.

<table>
<thead>
<tr>
<th></th>
<th>PN20</th>
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<th>PN22</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>E₀</td>
<td>Kₛ</td>
<td>%P</td>
<td>E₀</td>
</tr>
<tr>
<td>Site I</td>
<td>1</td>
<td>-2.23</td>
<td>2.29x10⁻²</td>
<td>24.62</td>
</tr>
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</tr>
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<td>9.13x10⁻³</td>
<td>21.82</td>
</tr>
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<td>18.89</td>
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<tr>
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<td>1.38x10⁻²</td>
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<tr>
<td></td>
<td>2</td>
<td>-3.46</td>
<td>2.90x10⁻³</td>
<td>17.57</td>
</tr>
</tbody>
</table>

KB is calculated in the equation \( K_b = \exp(\Delta G^{\text{1000}}/(R_{cal} \cdot T)) \), where \( \Delta G \) is the docking energy, \( R_{cal} \) is 1.98719 and TK is 298.15
PN22 on Site II is energetically weaker than the one observed on Site I. In Table 7.2 the two most populated families of the complexes of PN20 and PN22 with the monomeric Aβ42 are summarized. PN22 showed lower binding energies, while site I was in general energetically preferred over site II. Figure 7.5B shows the interacting complex of PN22 on Site I. Amongst all the interactions observed here, it is important to highlight that the NH3+ group of residue Lys forms a strong salt-bridge interaction with residue Asp39.

Pentameric Model. The target model used here was previously reported by Masman and coworkers (Masman et al. 2009a). This Aβ42 aggregates model contains two β-sheet moieties (β1, residues 18-26 and β2, residues 31-42), which are proposed as putative binding sites. Moreover, a third possible site for interactions with ligand molecules is postulated, which involves both above mentioned sites (β1 and β2) at the edge of the aggregate. This site, here called the TOP site, is orientated into the oligomeric axis, in the same direction where the oligomers grows by aggregation. Table 7.2 shows the two most populated families of the complexes of PN20 and PN22 with the pentameric Aβ42. It is observed in Figure 7.4B that PN20, in contrast with our expectation, has no preference to bind any of the sites targeted here. PN20 unselectively binds to β1, β2 and TOP sites. On the other hand, PN22 displayed a preference to bind the β2 site compared to the other two proposed sites. Figure 7.4C shows that the most populated conformations are energetically preferred when PN22 binds to site β2, while this peptide binds nonspecifically to β1 and TOP sites. Figure 7.6A illustrates an ideal H-bonding arrangement for TOP interaction of PN22 with an Aβ aggregate. It is depicted that PN22 may expose a “rich H-bonding face” to interact with the target molecule while exposing a “poor H-bonding face” that may stop the aggregation process. Figure 7.6B shows the atomic details of the interactions of the most populated complex (family 1 TOP, in Table 7.2). This interacting complex is the most similar arrangement to the ideal case. Even though the quantity of H-bonds was less numerous than in the ideal case, the orientation of the “poor and rich” H-bonding faces appear similar. Figure 7.7 reveals the atomic details of the interactions of the second most populated complex (family 2 β2, in Table 7.2) found between PN22 and the pentameric Aβ42. All the ligand-target contacts are depicted as wireframe spheres. PN22 is located in a transversal direction to the Aβ chains, and inside an important channel-like hydrophobic pocket formed between the residues Ile31 and Met35 of the aggregate. The side-chains of PN22 are localized parallel to the Aβ backbone. Thus, the side-chains act as hydrophobic anchors. Obviously, due to the highly hydrophobic nature of both, target and drug molecules, the predominant interaction type is the hydrophobic contact. Also, it is interesting to mention that the N-methyl groups face the Aβ aggregate, this way increasing the hydrophobic contact between drug and receptor. Accordingly, the “rich H-bonding face” is exposed to the surrounding space, where the solvent (water) should be.
**Figure 7.6:** (left) (A) An ideal TOP interaction of PN22 with an Aβaggregate. The N-methyl amino acid residues are shaded in grey. Hydrogen bonds are represented as slashed lines ---. (B) A stereoview of most populated complex PN22/Aβpentameric model on site TOP. All ligand-target contact are depicted as wireframe spheres.

**Figure 7.7:** (down) A stereoview of the second most populated complex PN22/Aβpentameric on site β2. Different views are shown: lateral (A), frontal (B), zoomed in (C and D). Panel C shows the ligand-target contact which are depicted as wireframe spheres. Panel D shows a stereoview of PN22 in the channel-like hydrophobic pocket formed, mainly by residues Ile31 and Met35.
7.3.2 Experimental testing

7.3.2.1 PN22 is neuroprotective against oligomeric Aβ_{42} in vitro

Part of this study was to provide proof of principle that the newly designed aggregation modulator peptides have the potential to protect nerve cells against Aβ-induced toxicity. Thus, we tested firstly whether these novel peptides exhibited any toxicity to neuronal cultures by themselves, and secondly whether PN20 and PN22 were capable to neutralize the toxic effect of oligomeric Aβ_{42}. To this purpose primary cortical neurons in culture were exposed to different concentrations of PN20 or PN22 alone, to Aβ_{42} alone (25 µM) or to pentapeptides and Aβ_{42} together for 24 h. Neither PN20 nor PN22 alone was toxic to neurons at any tested concentration (Figure 7.8). When exposed to 25 µM oligomeric Aβ_{42}, only 30-40% of the cultured neurons survived a 24 h incubation. Furthermore, both PN20 and PN22 were able to protect the neurons from Aβ_{42}-induced toxicity in a dose dependent manner (Figure 7.8). The methylated PN22 (Figure 7.8B) in that respect proved to be more effective reaching significance at 8 µM; P=0.02 when compared to the non-methylated PN20 (Figure 7.8A) (reaching significance at 32 µM; P=0.01). With the highest concentration (250 µM) of PN20 up to 62.3±12.9% of the neurons survived the Aβ treatment. 250 µM of PN22 was even more protective against the Aβ_{42} challenge with up to 86.3±3.7% cell survival.

7.3.2.2 Cognitive deficits induced by oligomeric Aβ_{42} can be neutralized by PN22

Based on the in vitro data we selected the most effective peptide PN22 and tested whether PN22 is able to reverse the Aβ-induced memory deficits. We further included a scrambled peptide (scrP) as a control for sequence specificity of the peptides. To that purpose we used an animal model in which we injected mice with oligomeric Aβ_{42} into the hippocampus to induce memory deficits (Granic et al., 2009).

One hour prior to the training session in a contextual fear conditioning paradigm C57BL/6J mice received a single injection of oligomeric Aβ_{42} (30 pmol) or vehicle with PN22 (150 pmol) or scrP (150 pmol). In general the injections did not affect locomotion or the shock reaction during training.

Figure 7.8: Cell viability of primary cortical neurons determined by an MTT-assay. Neuronal survival was determined after incubating increasing concentrations of the pentapeptides (A) PN20 and (B) PN22 with or without 25 µM Aβ_{42} for 24 h. Bars indicate the mean cell viability in % relative to untreated controls ± SEM. (*) = significant at p < 0.05; ** = highly significant at p<0.005).
The vehicle injected animals displayed an average relative freezing behavior, as a measure of memory score of 57.1± 2.2%, which did not differ from untreated control animals (58.1± 3.6 %). The injection of 30 pmol oligomerized Aβ42 led to a significant decrease in freezing behavior compared to the vehicle group (33.7± 6.5% vs 57.1± 2.2; p=0.002) (figure 7.9) indicative of impaired memory storage.

The PN22 and the scrP injected mice showed an average freezing of 54.9± 5.0% and 63.0± 5.5% respectively, which did not significantly differ from the untreated control (61.1± 2.4%) or vehicle injected group (58.9± 2.9%).

However, PN22 co-injected with Aβ42 was able to abolish the Aβ42 oligomer-induced memory impairment (53.7± 2.8% vs 33.7± 6.5%; p=0.01). Co-injection of Aβ with scrP resulted in an average freezing score of 48.0± 4.9%, which did not significantly reverse the Aβ-induced memory deficits. These results provide evidence that PN22 is capable to neutralize the negative effects induced by Aβ42 oligomers on memory performance in an in vivo test paradigm.

**Discussion**

Crucial for the aggregation process of the Aβ molecule are the hydrophobic residues that encompass the β2 region (amino acids 30-42). Thus, it was also observed for Aβ42 monomers in solution that the sequence I31IGLMVGGVIA42 (namely, the β2 portion) may be responsible for the higher propensity of this peptide to form amyloid aggregates (Sgourakis et al. 2007). Therefore, small peptides like PN20 and PN22, which are partially homologous to this hydrophobic region, bind with a relatively high affinity to Aβ by similar intermolecular interactions, leading to a competitive replacement of Aβ molecules. Interestingly our docking results showed that PN22 binds preferably to the β2 portion of the aggregate (table 7.2 and figure 7.4C), which has a good correlation with the design of this pentapeptide. However, in contrast with our expectations, PN20 did not show such a binding profile. This may be explained by the fact that PN20 displayed a complete different conformational behavior in solution than PN22 as shown by EDMC and MD simulations. PN22 had a strong preference for extended conformations, while PN20 showed a strong affinity to folded conformations. This was not only observed in solution but also in our docking results. Thus, an extended or semi-extended conformation was observed for the majority of the most populated complexes that PN22 formed, whereas the opposite was the case for...
PN20. It appears to be that an extended or semi-extended conformation is the possible “biologically relevant conformation” or “pharmacophoric patron” for these peptides.

On the other hand, all the docked preferred conformations showed a tendency to extended or semi-extended ligand’s orientation. Moreover, the docking studies could predict that a modification of PN20 by alternated N-methylation, thereby moderately increasing its hydrophobicity, improves the interaction to the β2 portion of the Aβ_{42} aggregates. Thus, PN22 shows a selective binding to the portion β2. Neither PN20 nor PN22 showed a preference to bind the monomeric or the pentameric Aβ_{42} model, which suggests us that the peptides may bind to both forms of Aβ_{42}.

We hypothesize that Site I-bound-PN22, especially on Asp^{23}, causes impediment or slowing down of the conformational change that precedes the aggregation. Consequently, PN22 binding increases the possibilities of degradation or clearance of Aβ monomers (see Figure 7.1), since, Aβ_{42} plays a crucial role in the stabilization of the Aβ aggregates. In general it can be observed that PN22 binds stronger and selectively to Site I, to the monomeric Aβ_{42} than PN20.

The present results indicated that pentapeptides PN20 and PN22 can significantly preserve cultured neurons from Aβ-induced cell death in a dose dependent manner. Moreover, the methylated pentapeptide PN22 protected the cells more effectively than the non-methylated PN20. Similar results on PC12 cells by using different single N-methyl amino acid containing peptides were previously reported (Cruz et al. 2004; Hughes et al. 2000). Peptides endowed with N-methyl amino acids have some clear advantages with respect to their potential as a therapeutic agent. These peptides remain remarkably stable to changes in solvent conditions and resist denaturation by heating, changes in pH (from 2.5 to 10.5), and addition of denaturants. Furthermore, this kind of peptides despite their hydrophobic composition is highly water soluble. A water soluble but hydrophobic nature suggests that these peptides might be able to pass spontaneously through biological cell membranes (Gordon et al. 2002).

An important aim of our study was to establish if a compound like PN22 is able to prevent Aβ-induced learning and memory deficits in a mammalian animal model. We showed that a 5-fold molar excess of PN22 to Aβ could overcome the detrimental effects of Aβ oligomers on memory when injected into the hippocampal region. Thus, we provide evidence that N-methyl containing peptides such as PN22 bear therapeutic potential against Aβ-induced memory impairment.

The mechanism how these pentapeptides exert their protective effects on cell death and behavior is not yet fully understood. However, these peptides directly bind to Aβ and thereby may prevent possible interactions between Aβ and neuronal membrane proteins and in this way neutralize the toxic effect of Aβ oligomers. We hypothesize two options of interaction notably of PN22 with Aβ that are depicted in figure 1. Pathway 1 proposes that PN22 binds to the monomeric Aβ thus preventing and/or retarding the formation of toxic oligomers, by interfering with the conformational change that precedes the oligomer formation. Pathway 2 suggests that PN22 binds to the already formed Aβ oligomers thereby preventing and/or modulating, somehow, its neurotoxic properties. By either of these actions, or both, the neurotoxicity of Aβ is decreased or reverted and the Aβ clearance/ degradation processes be reactivated.

We may conclude that this novel in silico designed N-methyl amino acid containing peptide interacts with Aβ_{42} and that different regions of Aβ may be selectively targeted by this peptide. Our findings provide evidence on how and where PN22 interacts with Aβ_{42} monomer- and oligomers, that PN22 can neutralize the neurotoxic effects.
of soluble Aβ$_{42}$ oligomers *in vitro* and *in vivo*. In the latter condition we obtained proof of principle that this pentapeptide can effectively prevent the Aβ oligomer-induced deficits in memory performance. More structural evidence is required to consolidate the proposed mechanism and to further improve our peptide designs. Nevertheless, it is clear that these peptides can serve as promising molecular designs that hold promise as therapeutic molecules for treatment of AD. ■

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“The brain may take advice, but not the heart, and love, having no geography, knows no boundaries: weight and sink it deep, no matter, it will rise and find the surface: and why not? any love is natural and beautiful that lies within a person’s nature; only hypocrites would hold a man responsible for what he loves, emotional illiterates and those of righteous envy, who, in their agitated concern, mistake so frequently the arrow pointing to heaven for the one that leads to hell.”
(T. Capote)