Peptide–Peptide Interactions

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Cognitive-Performance Recovery of Alzheimer's Disease Model Mice by Modulation of Early Soluble Amyloidal Assemblies**

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Alzheimer's disease (AD), a neurodegenerative disorder for which there is no disease-modifying therapy, is the foremost cause of dementia in elderly people.[1] An initial hypothesis suggested that fibrillar forms of β-amyloid polypeptide (Aβ) were responsible for neuronal dysfunction, but recent studies indicate soluble Aβ oligomers as the major toxic species.[2–4,6] An attractive therapeutic strategy for AD is to block the oligomerization of soluble amyloid (Aβ) peptides.[5–8] Herein we describe a novel oligomerization inhibitor whose mechanism of action is based on the targeting of aromatic recognition modules together with a unique C^{α}-methylation β-breakage strategy.[9] This small molecule interacts with early intermediate assemblies of Aβ and inhibits their assembly into toxic oligomers. NMR spectroscopy indicates that the inhibitor interacts with the aromatic core of Aβ. The orally bioavailable compound is very safe and reduced the amount of amyloid deposits in the brain of AD model mice. Treatment with this novel compound led to the recovery of the cognitive performance of model mice to the level of nontransgenic mice.

Amyloid deposits are associated with some of the major maladies of the 21st century. AD is one of more than 20 amyloid-related human disorders and is estimated to affect over 15 million people worldwide.[1] This figure is projected to double every 20 years as a result of an increasing life span.[1] Despite the grave implications of AD, no disease-modifying therapy exists; the only available treatment is symptomatic with limited effect. Although the etiology of AD is not fully understood, there is accumulating evidence that early oligomeric assemblies, rather than the hitherto suspected mature fibrils, reduce the function of cerebral neural cells as a result of their effect on key learning and memory functions, such as long-term potentiation (LTP).[2–4,10–15] Recently, dodecameric oligomers of Aβ were shown to be correlated with memory impairment in model AD mice[4] and to affect LTP activity.[3] Intriguingly, such dodecameric assemblies were found in extracts of human AD brain and not in control brain.[2,6] Most importantly, the synthetic oligomers and AD-derived oligomers from brain extracts were indistinguishable in terms of their structure, mass, and pI values, recognition by conformation-sensitive antibodies, and their selective attachment to the surface of nerve cells.[2] Soluble amyloid assemblies have also been identified in other degenerative amyloid-associated diseases.[8,11–12,15] Thus, the targeting of early molecular-recognition and self-assembly processes appears to be more promising for the treatment of AD and other amyloid-associated diseases than the disassembly of formed mature amyloid fibrils.[5–8]

We and others have identified the key role of aromatic residues in molecular-recognition and self-assembly processes to form various amyloid assemblies.[16–22] The targeting of the aromatic recognition interfaces was proposed to inhibit the very early steps of amyloid formation,[23–25] a hypothesis strengthened by theoretical and high-resolution structural studies carried out by other research groups, who had suggested aromatic interactions as a target for antiamyloid therapy.[26–30]

The use of β-breaker elements conjugated to recognition elements has been suggested as another way to inhibit amyloid formation.[31–33] A pioneering study by Soto et al. demonstrated the potential use of proline, the most effective naturally occurring β-breaker amino acid, for the design of novel pentapeptide inhibitors of AD Aβ polypeptide assemblies.[31,32] These inhibitors proved effective both in vitro and in vivo, yet their potential as drugs was limited by their relatively large size, peptide nature, and lack of oral availability. We recently introduced a novel approach for breaking β sheets by using α-methylated amino acids as higher-potency breaker elements.[9] Although the β-breaker strategy was suggested for inhibiting the formation of mature fibrils, it is also valid for inhibiting the formation of early intermediates, which are also rich in β-sheet structures.[9]

Small molecular inhibitors of amyloid formation that both target aromatic recognition interfaces and contain β-breaker elements were developed by performing iterative selection cycles on a library that combined aromatic recognition motifs and β-breaker motifs (see Figure S1 in the Supporting Information). A total of 40 rationally designed small molecules were screened in oligomer-inhibition assays based on thioflavin T (ThT) fluorescence. The results showed that a potent peptide inhibitor must contain a β-breaker element in the C terminus. The inhibitor must also contain metabolically stable D-amino acids to increase its potential as a drug lead.

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There is a preference for the noncoded amino acid α-aminoisobutyric acid (Aib), which has a much stronger β-breaking potential than proline[9] (see Figure S1 in the Supporting Information).

The chosen lead compound was a small dipeptide, NH_2-D-Trp-Aib-OH (Figure 1a; see also the Supporting Information). This dipeptide combines an indole, which we have identified as a potent aromatic binder of Aβ,[25] and α-aminoisobutyric acid (Aib). Although the compound was designed initially by the principles of peptide chemistry, it overcomes many limitations of previous peptide-based amyloid inhibitors. By virtue of its physicochemical and pharmacokinetic properties, as described below and in the Supporting Information, it has many characteristics of an ideal small-molecule drug, with a molecular weight of 289 Da, high serum stability, oral bioavailability, low toxicity, high solubility, and chemical stability in solution.

D-Trp-Aib targets the stage at which $A\beta_{1-42}$ assembles into toxic oligomers, as shown by using the protocol established by Hillen and co-workers.[3] This protocol gives sodium dodecylsulfate (SDS) stable oligomers that show toxic effects on the LTP of neural cells.[3] The effect of D-Trp-Aib on the formation of toxic polypeptide assemblies was assessed by incubating it with the polypeptide $A\beta_{1-42}$ at increasing molar ratios and examining the reaction mixtures by SDS-PAGE (Figure 1b). The results show a dose-dependent effect of D-Trp-Aib on the ability of early nontoxic intermediate oligomers (ca. 18 kDa) to grow further into toxic globulomer assemblies (ca. 56 kDa). The SDS-PAGE method enabled both the determination of the significant reduction in the amount of LTP-affecting globulomer structures formed and the establishment of the molecular identity of the $A\beta_{1-42}$ species formed. The dipeptide seems to stabilize the nontoxic early oligomers and inhibit their growth into toxic globulomers. The inhibitory effect of D-Trp-Aib was observed at a low molar ratio of 1:1; however, the inhibition profile is nonlinear. The decreased effect at mid-range molar ratios may be due to a competing homomolecular noncovalent interaction, as observed for various indole moieties The high concentrations of D-Trp-Aib are only relevant to the protocol as established for the rapid formation of oligomers at millimolar concentrations, and not for the physiological levels of Aβ or the envisioned therapeutic concentrations of D-Trp-Aib. The inhibitory effect on the formation of toxic globulomers from early intermediates appeared to be long-term, and the same level of inhibition was observed after incubation for 1 month (see Figure S2 in the Supporting Information).

The affinity of D-Trp-Aib for the early intermediates was shown in fluorescence anisotropy experiments, in which we took advantage of the intrinsic fluorescence of D-Trp-Aib and its small size relative to that of the $A\beta_{1-42}$ oligomers. Increasing amounts of the early intermediate $A\beta_{1-42}$ were titrated into a solution of D-Trp-Aib, and the anisotropy of the solution was determined (Figure 1c). The affinity of D-Trp-Aib for $A\beta_{1-42}$ was found to be about 300 nM. Although this figure is higher than typical values for receptor–drug affinity, this degree of affinity is quite efficient for agents that target recognition interfaces. The low toxicity of D-Trp-Aib will enable its potential use in vivo despite its binding affinity.

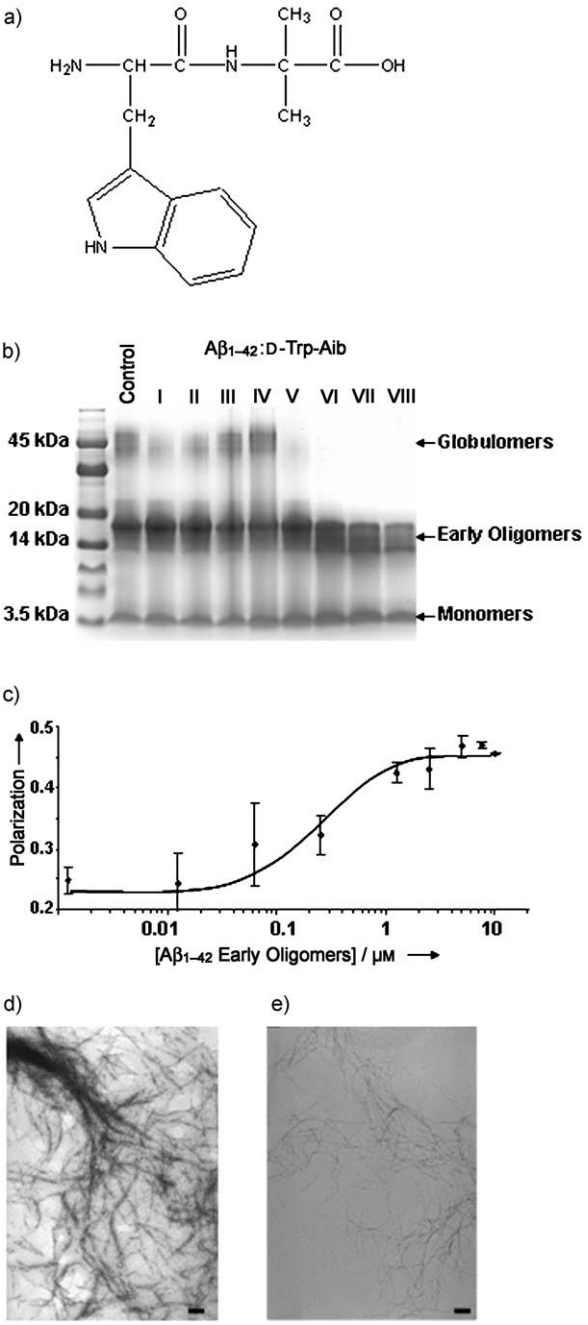

Figure 1. Inhibition of oligomer assembly by D-Trp-Aib. a) Structure of D-Trp-Aib. b) Determination of the dose-dependent effect of D-Trp-Aib on the formation of soluble oligomers. Soluble oligomers were prepared according to the method of Barghorn et al. in the presence of an increasing amount of D-Trp-Aib and without D-Trp-Aib (the control is $A\beta_{1-42}$ only): I) 1:1 ($A\beta_{1-42}$:D-Trp-Aib), II) 1:5, III) 1:10, IV) 1:20, V) 1:40, VI) 1:60, VII) 1:80, VIII) 1:100. c) The affinity of D-Trp-Aib for early oligomers was determined by fluorescence anisotropy. d) TEM micrograph of a control sample containing $A\beta_{1-42}$ in phosphate-buffered saline after incubation for 7 days. e) TEM micrograph of a sample containing $A\beta_{1-42}$ in the presence D-Trp-Aib (1:2 molar ratio). Scale bar: 200 nm.

Transmission electron microscopy (TEM) was used to determine the effect of the inhibitory compound on the ultrastructural properties of assembled $A\beta_{1-42}$. The fibrils

formed by the samples containing D-Trp-Aib (present in twofold excess) were thinner, smaller, and fewer than those with $A\beta_{1-42}$ only (Figure 1d,e). These findings also show that the short peptide affects the very early stages of fibrillation and prevent it from growing into the toxic species.

We assessed the effect of D-Trp-Aib on the toxicity of early intermediates by the MTT assay (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). D-Trp-Aib was found to inhibit significantly the toxic effect of $A\beta_{1-42}$ early assemblies towards PC12 cell cultures, with a maximum effect when D-Trp-Aib was present in 10-fold excess (Figure 2a).

Fluorescence-activated cell sorting (FACS) analysis was performed on a PC12 cell line (Figure 2b–d). Cells treated with $A\beta_{1-42}$ early assemblies were most commonly in the early and late apoptotic phase (Figure 2b,c), whereas cells treated with both early assemblies and D-Trp-Aib (40-fold excess) exhibited a much higher degree of viability and less apoptosis (Figure 2b,d). These results are consistent with the MTT assay. They reveal the protective effect of the D-Trp-Aib inhibitor against the toxicity of the early assemblies and indicate clearly that the compound prevents apoptotic death, which is most likely relevant to the pathological state.

The D-Trp-Aib–Aβ interaction was investigated by NMR spectroscopy. Aβ residues 16–22 have been shown to participate in the transition into the β-sheet secondary structure and are independently capable of forming amyloid fibrils.[34–36] A longer non-aggregative fragment, $A\beta_{12-28}$, was used to prevent complications from oligomerization processes. The spectrum of a sample containing a fourfold excess of D-Trp-Aib with $A\beta_{12-28}$ (Figure 3a) was compared to spectra of the individual components and showed deviations in the chemical shifts of the backbone amide hydrogen atoms of more than 0.04 ppm upon the binding of residues Val18, Glu22, Asp23, and the terminal Lys28 residue. The residues from Val18 to Ser26 showed higher deviations in the chemical shifts of the amide hydrogen atom than the rest of the molecule, except Phe20, which showed a significant chemical-shift deviation for the α hydrogen atom. The $^3J_{HN,H^\alpha}$ coupling values showed deviations of above 0.6 Hz for residues Phe20, Asp24, Val24, and Asn27; other residues from Leu17 to Lys28 showed deviations of less than 0.1 Hz, and the first three N-terminal residues showed deviations of 0.2–0.4 Hz (Figure 3a,b). The chemical shift of D-Trp-Aib itself showed negligible deviations upon binding as a result of its presence in excess relative to the peptide to favor maximum interaction.

The solution structure of Aβ bound to D-Trp-Aib shows a loose turn in the Phe20–Asp23 region, the existence of which is supported by NOE connectivities, low values of the four-residue local root-mean-square deviation (RMSD) in the region of Val18–Asp23, and a geometry conducive to possible backbone hydrogen bonding (Figure 3b). The solution structure and the electrostatic potential of the peptide when bound to D-Trp-Aib is given for 20 low-energy structures (RMSD residues 16–23, 1.22 Å (backbone) and 2.62 Å (heavy atoms; Figure 3c,d).

A single symmetrical peak corresponding to an intermolecular interaction between the α hydrogen atoms of Ala21 and the β hydrogen atoms of the Aib residues in D-Trp-Aib

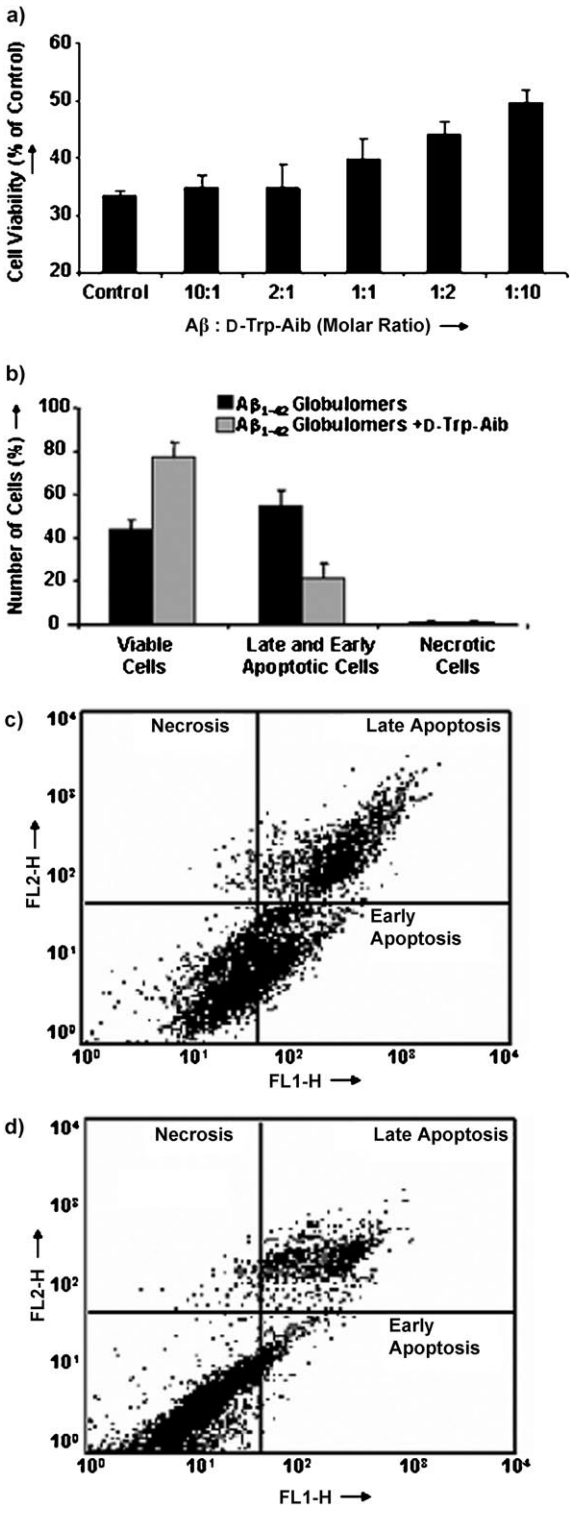

Figure 2. Cell culture experiment. a) Effect of D-Trp-Aib on the toxicity of soluble oligmers. Soluble oligomers were prepared according to Barghorn et al. without D-Trp-Aib and with increasing amounts of D-Trp-Aib. The cytotoxic effect on PC12 cells was determined by using the MTT assay. b) Quantitative analysis of the FACS results. c) FACS results for the incubation of PC12 cells with an early intermediate alone. The annexin V-FITC apoptosis detection kit was used for the detection of apoptotic cells. d) FACS results for the incubation of PC12 cells with an early intermediate and D-Trp-Aib in a 1:40 molar ratio. The annexin V-FITC apoptosis detection kit was used. FL1-H is the fluorescence of V-FITC and FL2-H is the fluorescence of annexin V-PE.

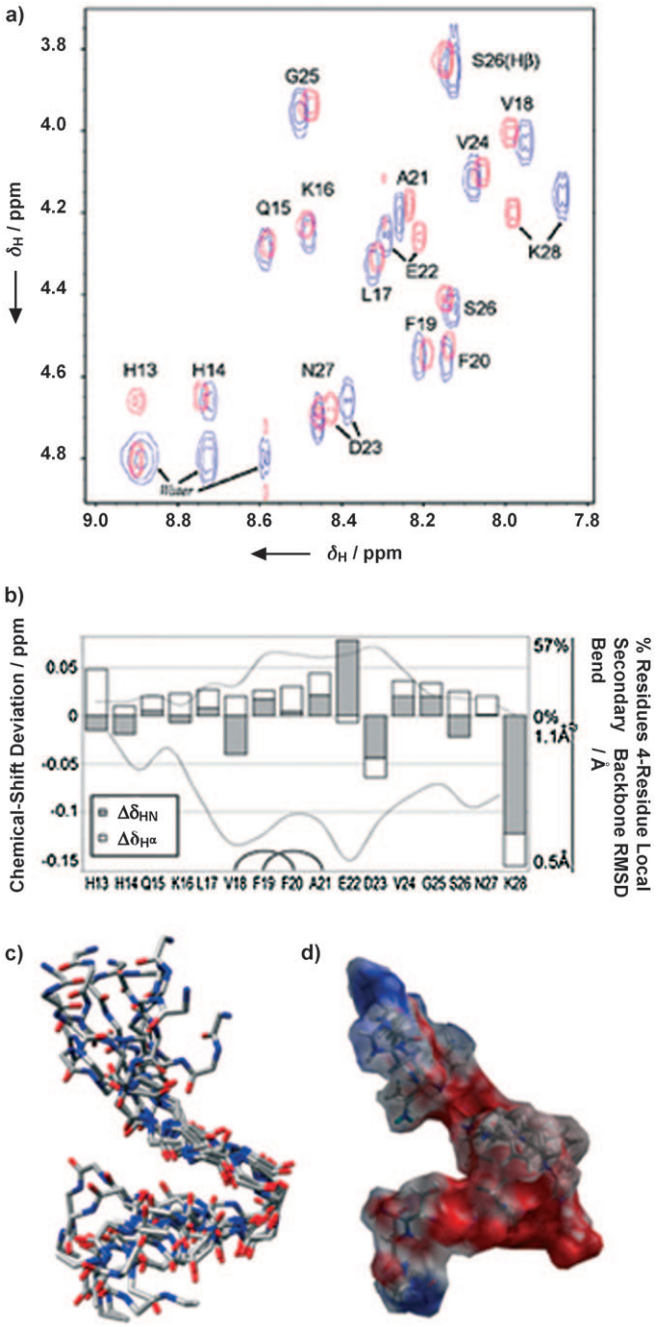

Figure 3. NMR spectroscopic analysis of the Aβ–D-Trp-Aib interactions. a) HN–H^{α} region of the TOCSY spectra of $A\beta_{12-28}$ bound to D-Trp-Aib (red) and when free (blue). b) NMR sequence-specific spectroscopic data. The diagram shows the HN (filled boxes) and H^{α} (open boxes) chemical-shift deviations upon the binding of $A\beta_{12-28}$ to D-Trp-Aib. The percentage of residues in bend conformations is shown in the upper curve, and the four-residue local backbone RMSD (Å) is shown in the lower curve. c) Ten low-energy conformations superimposed in the turn region (residues 16–23; RMSD: 1.00 Å (backbone), 2.62 Å (heavy atoms)). d) Electrostatic potential mapped on the van der Waals surface of the low-energy conformation. (Molecular graphics images were produced by using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081).)

was identified in the TOCSY spectrum of D-Trp-Aib–Aβ. Although no further intermolecular interactions were detected, the molecular structure, together with the chemical-shift deviations, provide strong evidence for the position of the interaction between D-Trp-Aib and Aβ in the vicinity of the central aromatic recognition module of the peptide. The interaction is likely to be hydrophobic in nature and augmented by an aromatic interaction between the aromatic rings of Phe19-Phe20 and D-Trp in D-Trp-Aib.

The effect of D-Trp-Aib on AD model mice was studied on a total of 23 transgenic mice (aged 4.5 months) that overexpress hAPP. Animals were treated for 120 days to enable the clear end observation of changes in plaque load and learning abilities. In general, all animals were able to fulfill the behavioral tasks. Results obtained on days 3 and 4 with the Morris water maze (MWM) showed significant differences between the group treated with D-Trp-Aib and the vehicle-treated group: The behavior of mice treated with D-Trp-Aib was improved significantly (Figure 4a,b). More importantly, the cognitive performance of transgenic (tg) mice treated with D-Trp-Aib returned to levels that were not statistically different from those of nontransgenic mice with the same genetic background. Treatment with D-Trp-Aib also led to a notable decrease in the concentration of Aβ in the brain of hAPP tg animals relative to the vehicle-treated control group (see Figure S4 in the Supporting Information). The mean size of the plaque cores in the cortex of D-Trp-Aib-treated animals was significantly smaller than in vehicle-treated controls (Figure 4c–h).

The safety and availability of D-Trp-Aib were tested to determine its potential as a drug lead. D-Trp-Aib was found to be completely nontoxic to cultured cells up to a concentration of 50 mM. Its interaction with the hERG potassium channel also suggested a favorable safety profile. Single-dose acute intravenous (i.v.) toxicity testing in mice showed that the MTD (maximum tolerated dose) of D-Trp-Aib is between 750 and 1000 $mg\,kg^{-1}$. All male and most female mice showed no symptoms upon i.v. administration of 1000 $mg\,kg^{-1}$ D-Trp-Aib. Therefore, although the formal MTD is 750 $mg\,kg^{-1}$, the MTD for male mice is $>1000\ mg\,kg^{-1}$, and in many cases no adverse signs are associated with the i.v. administration of 1000 $mg\,kg^{-1}$ of the dipeptide to female mice. The compound also showed no signs of genotoxicty in an Ames test with five bacterial strains. Thus, D-Trp-Aib is likely to be a very safe compound.

We determined the stability and pharmacokinetic profiles of D-Trp-Aib. Stability is a key issue for the use of peptide-based therapeutic agents. D-Trp-Aib showed complete stability in both mouse and human serum for 24 h, as expected for a dipeptide composed of a D isomer and the nonchiral amino acid Aib. The stability of D-Trp-Aib spiked into serum was evaluated by analyzing the concentration of intact sample at different time intervals. No decrease in the concentration of D-Trp-Aib was observed after incubation for 2 or 5 h with mouse or human serum.

Oral availability and the ability to cross the blood–brain barrier (BBB) are important properties for peptide-based drugs. The serum-stable compound D-Trp-Aib was administered to mice by either i.v., oral, or intranasal routes, and the

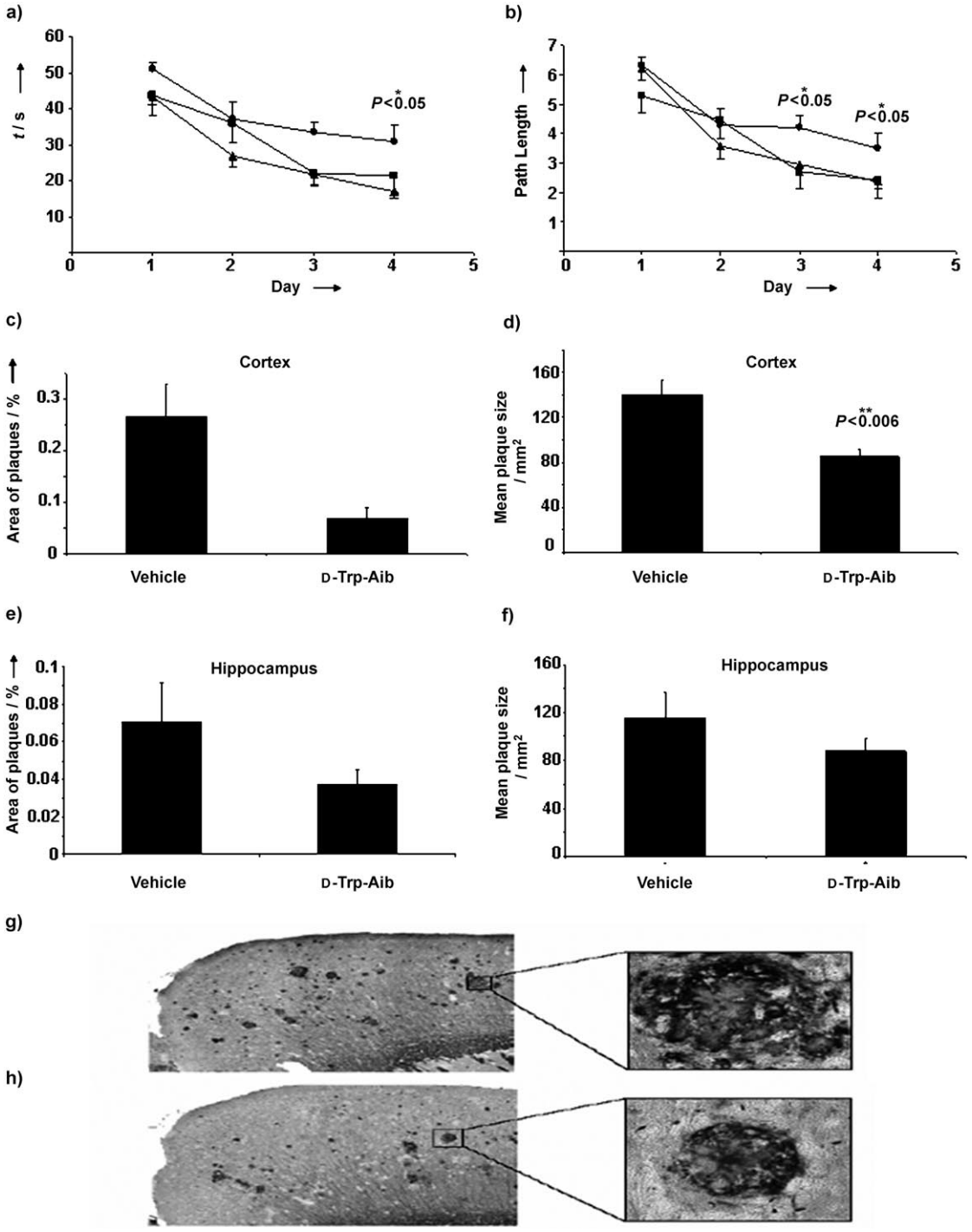

Figure 4. Effect of D-Trp-Aib on the cognitive performance (determined by using the Morris water maze (MWM)) and plaque load in Alzheimer's disease model mice. Mice were trained for 2 days, and their performance in reaching the platform was determined on the basis of a) time and b) the path length swum (● vehicle-treated AD model mice, ■ AD model mice treated with D-Trp-Aib, ▲ nontransgenic mice). c),e) The relative plaque-load area of hAPP tg mice treated with D-Trp-Aib and vehicle-treated hAPP tg mice was determined by labeling with thioflavin S; d),f) the mean plaque size was also determined. g) Histological images from cutouts of the frontal cortex of a vehicle-treated group (stained with the Campbell–Switzer stain). h) Cutouts from the frontal cortex of a D-Trp-Aib-treated group. Data are presented as the mean ± SEM (standard error of the mean). The normal distribution of measurement values was tested with a Kolmogorov-Smirnov test. Group differences were calculated by a two-tailed T-test or a non-parametric Mann-Whitney U-test, if data were not normally distributed

amount of compound was determined in mice by LC–MS/MS analysis (see the Supporting Information). The compound was expected to show some oral bioavailability according to Caco2 experiments (see the Supporting Information). However, it also showed excellent bioavailability upon both oral (39%; nonoptimized formulation) and nasal (55%) administration. Despite its excellent absorption by the digestive tract, the compound was highly water-soluble at concentra-

tions as high as 100 mg mL^{-1} in aqueous and saline solutions. The percentage of the compound that crossed the blood–brain barrier was in the range of 4–8%, depending on the route of administration.

In summary, we have described a novel inhibitor of the oligomerization of β amyloid polypeptide. The compound combines the targeting of aromatic recognition interfaces with a novel C^{α}-methylation β-breakage strategy. The small-molecule (289 Da) inhibitor interacts specifically with early low-molecular-weight soluble assemblies of the Alzheimer's disease β amyloid polypeptide and inhibits their toxicity towards cultured cells. The compound is orally bioavailable and very safe in vitro and in vivo. It reduced the amount of amyloid deposits in the brain of AD transgenic mice models significantly, and improved their cognitive performance. The ability of the compound to restore cognitive performance in AD transgenic mice further suggests that the targeting of early oligomers is a promising strategy for the treatment of Alzheimer's disease.

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