Blood-brain barrier penetration of an Aβ-targeted, arginine-rich, D-enantiomeric peptide

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Abstract

The application of small peptides targeting amyloid beta (Aβ) is one of many drug development strategies for the treatment of Alzheimer’s disease (AD). We have previously identified several peptides consisting solely of D-enantiomeric amino acid residues obtained from mirror-image phage display selection, which bind to Aβ in different assembly states and eliminate toxic Aβ aggregates. Some of these D-peptides show both diagnostic and therapeutic potential in vitro and in vivo. Here we have analysed the similarity of the arginine-rich D-peptide D3 to the arginine-rich motif (ARM) of the human immunodeficiency virus type 1 transactivator of transcription (HIV-Tat) protein, and examined its in vivo blood-brain barrier (BBB) permeability using wild type mice and transgenic mouse models of Alzheimer’s disease. We are able to demonstrate that D3 rapidly enters the brain where it can be found associated with amyloid plaques suggesting a direct penetration of BBB.

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1. Introduction

Currently, two molecular weight categories classify drugs into conventional “small molecule” drugs (< 500 Da) and “biologics” which are generally referred to as protein-based drugs (> 5000 Da) [1]. Small molecules are often associated with favorable oral bioavailability and their production is considered scalable and economical. During drug discovery, they can be rationally designed, for example, altering the structure to optimize physiochemical properties for enhanced brain delivery [2, 3]. Frequently, however, small molecules show low target selectivity, which may ultimately result in side effects. Protein-based drugs, for example antibodies, possess high selectivity, mainly because their large size allows formation of specific and high affinity binding sites for their target molecules. Usually they have poor oral bioavailability due to low membrane permeability and proteolytic instability [4]. Small peptide based molecules consisting of 5 to 50 amino acid residues may fill the molecular weight gap and combine the advantages of small molecules and protein based drugs.

Well-known examples of peptide-based drugs with high medical and economic impact are the peptide hormones insulin and glucagon. Diseases like diabetes, cancer, inflammation and cardiovascular diseases, are strong drivers for the development of peptide based drugs [5]. Peptide drugs have the potential for high substrate specificity and affinity. Their degradation usually doesn’t lead to toxic metabolites. They are smaller than proteins and thus can be obtained synthetically by well-established and cost-efficient methods [6]. A drawback of peptide drugs is their relatively low bioavailability due to degradation and the resulting short half-lives. Several approaches have been developed to enhance the bioavailability of peptide based drug candidates. The application of D-enantiomeric amino acids is an effective way to enhance the resistance to degradation, because most proteolytic enzymes have affinity and high af

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levels of efflux transporters at their luminal endothelial surface. This efficiently limits the penetration potential of 95% of all known drugs into the brain [12]. In the absence of active transport mechanisms, the ability of peptides to permeate through membranes usually decreases with increasing mass and hydrophobicity. Studies have shown that most drugs with molecular weights above 500 Da already show poor brain penetration potential and compounds with molecular weights above 1000 Da are usually widely excluded from the passive transmembrane transport system [13,14]. In spite of poor BBB permeability in general, some peptides can be transported into the brain via specific transporters expressed in brain endothelium under physiological or pathological conditions [15,16]. Furthermore, several BBB penetration mechanisms such as receptor-mediated, adsorptive-mediated or carrier-mediated mechanism are intensively studied [17]. Properties such as the presence of basic clusters have been found to trigger the uptake of compounds into the cell by an as yet unresolved mechanism. A well-known example is the human immunodeficiency virus type 1 (HIV-1) transactivator of transcription (Tat) [18–20]. The HIV-1 Tat is a regulatory protein with 86 to 101 amino acids depending on the subtype. It is produced in the very early stages of viral infection and greatly enhances the transcriptional rates which result in high viral gene expression. It also mediates viral spreading in disease progression [21]. The basic region of HIV-1 Tat protein involved in RNA binding is rich in arginines and lysines, and thus belongs to the family of arginine-rich motif (ARM) RNA binding proteins [22]. Such ARMs have been identified first in lentiviral Tat proteins, e.g. from HIV and equine infectious anaemia virus (EIAV), and later in a variety of nucleic acid binding proteins. Functional and structural details have been described for Tat proteins [23–26]. Currently there are several hypotheses about the potential mechanism for HIV-1 Tat uptake: these all incorporate the fact that HIV-1 Tat binds negatively charged targets such as heparane sulfate/glycosaminoglycans, sialic acid and phospholipids, and traverses the plasma membranes passively. Furthermore, recent in vitro experiments based on artificial membrane systems suggest the formation of plasma-membrane pores [18–20,27]. The exact mechanism of ARM transduction is still unknown, but the relative abundance of arginines is suggested to play a decisive role [28,29]. Although lysine presents the same positive net charge as arginine, the substitution of arginines with lysines decreases the transduction efficiency [30,31]. Studies show that arginine-rich peptides, which demonstrate membrane permeation, lack secondary structure, and D-enantiomeric arginine as well as guanidino peptides work equivalently [28,31,32], which demonstrate that the guanidinium groups are the critical structural component responsible for the transduction [28,30,33]. Such mechanisms may be beneficial for peptide design.

We have identified several D-peptides by mirror-image phage display [34] for binding to Aβ [35,36]. They show promising abilities, e.g. elimination of Aβ oligomers and inhibition of Aβ fibril formation in vitro [37], and fluorescein labelled D-peptides bind to amyloid plaques in transgenic mice after direct brain infusion. [38]. One of those α-peptides, D3, was able to reduce plaque load and inflammation markers in the brains of aged APP/PSE double transgenic mice and select peptide-based drugs and suggests that D3 and its derivatives are promising candidates for the future treatment of Alzheimer’s disease.

2. Materials and methods

2.1. Peptides and other chemicals

3H-D3 (rprtrlhthrnr) was purchased from Quotient Bio research (Radiochemicals) Ltd. (Cardiff, United Kingdom) with 10–100 Ci/mmol, 1 mCi/ml and purity >95%. D3 (rprtrlhthrnr), FAM-D3 (H-rprrtlhthrnr-Lys(5(6)-carboxyfluorescein)-NH2) and FAM-LP (Lrmmrlrhlthrnr-Lys(5(6)-carboxyfluorescein)-NH2) was purchased from JPT peptide Technologies GmbH (Berlin, Germany). Molecular weight of D3 is 1599, while FAM-D3 is 2086; The FAM-tagging through 5-FAM lysine introduced one negative charge to D3 from a carboxylic acid at 5-FAM molecule. Thioflavine S was from Sigma-Aldrich (Munich, Germany). All other chemicals were supplied by Fluka Chemie AG (Buchs, Switzerland), Merck (Darmstadt, Germany), AppliChem (Darmstadt, Germany) and VWR (Darmstadt, Germany) in research grade.

2.2. Animals

To study the distribution of 3H-D3, male C57Bl/6 mice (Charles River, Sulzfeld Germany) with an average body weight of 33.4 g were used. The mice were hosted in our animal facility under standard housing conditions for at least 2 weeks before experiment. All animal experiments were approved by the Animal Protection Committee of the local government (LANUV, North-Rhine-Westphalia, Germany, AZB4-02-04.2011. A359 and AZB4-02.04.2011. A356) according to the German Protection of Animals Act.). For the experiment of FAM-D3 oral administration, seven male four-month old APP and PS1 double transgenic mice (APPSwe/PS1ΔE9) were used in the present study. It has been shown that animals from this line exhibit numerous plaques and cerebral amyloid angiopathy within the brain, especially within cortex and hippocampus but also in other brain areas including the cerebellum [47–49]. The APPswe/PS1ΔE9 mice were acquired from JAX at the age of six weeks and housed in 4/cage in a controlled environment (temperature 22 °C, humidity 50–60%, and light from 07:00–19:00) until the treatments; food and water were available ad libitum. The experiment was conducted in accordance with the local Institutional Animal Care and Use Committee (IACUC) guidelines. All animal studies comply with the ARRIVE guidelines [50]. A completed ARRIVE guidelines checklist was included in S1 ARRIVE Checklist.

2.3. 3H-D3 distribution in brain, plasma and CSF

3H-D3 was mixed with non-radioactive D3 in a 0.1 M phosphate buffer (pH 8) to a total D3 concentration of 3 mg/ml with a specific radioactivity of 16.7 μCi per mg D3. 5 μCi D3 (100 μl) were administered as a single bolus intraperitoneally. Doses were selected according to

HIV-1 (HXB2) Tat 53-64: RQRRARHCONSQT
D3 1-12: rprtrlhthrnr

Fig. 1. Alignment of D3 with human immunodeficiency virus type 1 (HIV-1), strain HXB2, transactivator (Tat) protein. D3 shows sequence similarity to the arginine-rich motif (ARM) of a recombinant inbred strain (HXB2) of HIV-1 Tat protein.

To test this hypothesis, we have studied the in vivo localization of D3 in the CNS and its potential to pass the BBB as well as to bind amyloid plaques in vivo and in vitro in mouse brain sections.

Our results suggest that the BBB penetration of D3 may indeed have the same mechanism as HIV-1 Tat. This may offer a strategy to design and select peptide-based drugs and suggests that D3 and its derivatives are promising candidates for the future treatment of Alzheimer’s disease.
tolerability studies and did not cause adverse reactions. The total D3 concentration was calculated through 3H radioactivity assuming radioactive/non-radioactive ratio of the working solution stays the same during bio-distribution. Sampling times were 30, 60, 240 and 1440 min after administration (3 mice per time point). The administration method and the sampling time were selected based on our previous pharmacokinetic study [7]. Mice were anaesthetized with ketamine/medetomidine 10 min before samples were collected. Cerebrospinal fluid (CSF) was collected with a glass capillary tube (Round Boro Capillaries, CM Scientific, New Jersey, USA) from the cisterna magna as described by Liu and Duff [51]. About 8 to 9 μl CSF was obtained from each mouse. Blood was drawn by heart puncture and heparinized to isolate blood plasma. The right brain hemisphere was isolated directly after euthanasia through cervical dislocation.

The brain hemisphere was weighed and homogenized in homogenizer tubes (Precellsy Ceramic Kit 1.4 mm, Precellsy 24, Bertin technol- ogy SAS, Montigny le Bretonneux, France) with 500 μl PBS. 10 ml scintillation cocktail (Ultima Gold XR, PerkinElmer, Waltham, Massa- chusetts, USA) was added to 100 μl of brain homogenate or plasma (diluted 1:1 with PBS) and mixed well. Disintegrations per unit time (dpm) were obtained in triplicates with a liquid scintillation counter (Packard Tri-Carb 2100TR Liquid Scintillation Analyser, PerkinElmer, Waltham, MA, USA). CSF was mixed directly with 10 ml scintillation cocktail.

Radioactivity was quantified in each sample as the percentage of injected dose (ID) per weight unit for brain tissue (%ID/g), or dose per volume unit for plasma/CSF samples (%ID/ml). Analogously, for cold material the concentration was expressed as weight unit of total D3 per weight unit of brain tissue (μg/g), or as weight unit per volume unit for plasma/CSF samples (μg/ml). Ratios were calculated from the values of brain, CSF and plasma, respectively. Mean values with standard error (SEM) of three mice were presented.

2.4. In vitro autoradiography (3H) with mouse brain sections

Brain from a homozygous 18.5 months old APP/PS1 (ARTE10) trans- genic mouse is a generous gift from Andre Manook (Nuklearmedizinische Klinik und Poliklinik, Klinikum rechts der Isar, Technische Universität München, Munich, Germany). Homozygous ARTE10 mice of this age exhibit extensive plaque load and to a lesser extent cerebral amyloid angiopathy especially in cortex and hippocampus, but also in other areas except the cerebellum [42,52]. The brain was fixed in 4% formaldehyde (Carl Roth, Karlsruhe, Germany) in PBS for one week at room temperature then transferred into 30% sucrose in PBS for 2 days at 4 °C. A series of 30 μm free floating cryosections were cut sagittal and post-fixed in 4% paraformaldehyde for 30 min at room temperature. After washing and 10 min permeabilization with 1% Triton X-100 in Tris-buffered saline (TBS), sections were transferred into a staining dish containing 3H-D3 solution in 1% Triton-TBS (2.5 k-becquerel (kBq) per section) and incubated for 3 days at room temperature. After washing in TBS and a rapid washing step in H2O, sections were mounted on glass slides and dried at 37 °C for 2 h, then exposed against a phosphor-imaging plate for 3H-autoradiography (FUJIFILM, Tokyo, Japan) in an autora- diography cassette for 7 days. The imaging plate was scanned with a phosphor film imager (Fujifilm BAS-5000, FUJIFILM Life Science, Japan) and images were acquired with BAS reader and AIDA software (Raytest, Freiburg, Germany).

2.5. Thioflavine S staining

Sections from in vitro autoradiography were immersion-fixed in 4% paraformaldehyde for 20 min, and then equilibrated in water twice for 2 min. After incubation in freshly filtered 1% (w/v in water) Thioflavine S for 30 min at RT in a dark chamber, sections were washed twice for 2 min in H2O, and differentiated in two changes of 80% ethanol for 5 and 1 min, respectively. After washing three times in water (2 min per iteration), sections were covered with a glass slide and Aqua Poly/ Mount (Polysciences, Warrington, US). Images were acquired with a Lumar V12 SteREO microscope (Zeiss, Oberkochen, Germany) with AxioCamMR3 camera (Zeiss, Oberkochen, Germany) and processed with AxioVs40 software (Release 4.5 SP1) and ImageJ (1.48 s).

2.6. Oral administration of D3 and FAM-D3

We chose oral administration for this long term experiments as it is favorable for animals. In order to achieve the desired total D3 concentration, non-labelled D3 (90%) was added to FAM-D3 (10%) with a final concentration of 0.25 mg/ml. Seven four-month-old APPsw/ePS1AΔE9 transgenic mice were treated for eight weeks with FAM-D3 and D3 in the drinking water. On average, the mouse drank ca. 2 to 3 ml water per day. The stability of D3 in water containing mouse saliva was verified using reversed phase HPLC analysis. Briefly, D3 was dissolved in double-distilled water, drinking water and drinking water containing 0.5% (v/v) mouse saliva to a final concentration of 27 μM. Freshly prepared solution and solution incubated for 24 h at 37 °C were analysed through reversed phase HPLC with a C18 column (Phenomenex, Aschaffenbourg, Germany) and compared. No obvious degradation was observed. Eight weeks after the start of treatment, the mice were sacrificed for histopathological analysis (see below).

2.7. Immunohistochemistry and immunofluorescence

Brain sections from the transgenic mouse orally administered with FAM-D3 were treated with a monoclonal mouse anti-Aβ(4–10) (W0–2) antibody (EMD Millipore). The sections were incubated overnight in a solution of TBS; then the sections were treated for 30 min in a heat- ed (85 °C) sodium citrate solution (0.05 M, pH 6.0) and allowed to cool down. Afterwards, the series of sections were transferred into TBS-T (TBS with 0.5% Triton X-100) containing the primary antibody (mouse anti-Aβ(4–10), USA) for 24 h at 20 °C in a dark room. The sections were washed three times in TBS-T and transferred into a solution containing the secondary antibody (biotinylated goat anti mouse; Sigma) for 2 h. Again, the sections were washed three times with TBS-T and transferred to a solution containing ExtrAvidin for 2 h. Then the sections were incubated for approximately 3 min with Ni-enhanced diaminobenzidine (DAB) (12.5 mg DAB in 25 ml 0.1 M phosphate buffer, pH 7.4, 30 μl H2O2 (30%), with 1 ml of a 15% ammonium Ni-sulfate solution added). The stained sections were mounted on gelatinized slides and coverslipped.

Cerebral blood vessels were visualized with GLUT-1 antibody (rabbit anti-GLUT-1 antibody EMD Millipore). The staining was performed exactly as described for Aβ, with the exception that no pretreatment was performed and a fluorescent secondary antibody (goat anti rabbit, Jackson ImmunoResearch) was applied. The stained sections were mounted on gelatinized slides and coverslipped.

2.8. Thin layer chromatography

Untreated wildtype mouse brain was homogenized with the same method as described above. After centrifuge at 20,000 × g for 15 min at 4 °C to clarify the homogenate, extract of mouse brain was obtained from supernatant. FAM labelled peptide was incubated with the brain extract at 37 °C for different time periods (from 0 to 2 days). 2 μg FAM labelled peptide was mixed with 1 μl brain extract (in excess to peptide [53,54]). Mixtures containing FAM-labelled peptides were applied onto a HPTLC cellulose plate (OMNILAB, Essen, Germany) for thin layer chromatography (TLC) with mobile solvent (2-butanol/pyridine/acetic acid/ water (30/20/6/24)). Images were acquired using the ChemDoc MP imaging system (Bio-RAD, Munich, Germany) under the fluorescein channel. The retardation factor (Rf) of each substance was defined as the ratio of the migration distance of the centre of a separated spot to the migration distance of the solvent front.
3. Results

3.1. Temporal distribution of $^3$H-D3 in brain, plasma and CSF after intraperitoneal administration

To study the temporal and spatial distribution of D3 in vivo, we have analysed the concentrations of radioactively labelled D3 in brain, plasma and CSF. The time-concentration profiles of $^3$H-D3 in whole brain, plasma and CSF after i.p. administration are shown in Fig. 2. Brain/plasma, CSF/plasma and brain/CSF ratios are shown in Fig. 3.

As expected, the highest concentration of D3 was measured in plasma shortly after administration with 8.4 μg/ml after 4 h and subsequently decreased to 0.53 μg/ml, whereas the concentration of D3 in brain and CSF started at about 0.6 μg/ml and decreased only slightly (Fig. 2). The brain/plasma ratio reached 0.8 to 0.9 after 4 h. D3 concentration in CSF remained slightly higher in comparison to that in the brain at all four time points, resulting in a relatively stable brain/CSF ratio of about 0.8 (Fig. 3).

3.2. Binding of $^3$H-D3 on Aβ plaques by in vitro autoradiography

In order to study the influence of $^3$H-D3 within cerebral blood vessels on the total radioactivity detected in the whole brain, brain slices of a transgenic APP/PS1 (ARTE 10) mouse were incubated with $^3$H-D3 and subsequently developed by in vitro autoradiography (Fig. 4A). Additionally, the same slices were stained with Thioflavin S to stain beta-sheet rich structure elements (Fig. 4B), which is regarded as a robust and easy method for Aβ plaque quantification in this mouse model [55]. Co-localization of $^3$H-D3 and Thioflavin S is an indicator of the specific binding of D3 to the amyloid plaques (Fig. 4C). Furthermore, the use of 1% Thioflavin S causes the strong non-specific staining of blood vessels [56], which also visualizes cerebral blood vessels on the section (Fig. 4D).

The APP/PS1 (ARTE 10) mouse model used is characterized by the lack of Aβ plaques in the cerebellum [55], which is in agreement with the Thioflavin S stain, as well as the autoradiography with $^3$H-D3 in this study. No $^3$H-D3 labelled structures of blood vessels in either the cerebrum or cerebellum could be observed in the autoradiogram, even though some of the vessels were clearly stained by Thioflavin S (Fig. 4D). This shows that $^3$H-D3 bound specifically to Aβ plaques in the parenchyma and any specific binding of $^3$H-D3 to cerebral blood vessels was negligible.

3.3. Oral administration of FAM-D3

As demonstrated recently in a pharmacokinetic study, oral administration yielded similar D3 concentrations after 4 h in the brain as i.p. and i.v. administrations did [7]. Here we used fluorescently labelled D3 for the purpose of direct visualization. After eight weeks of oral administration of S(6)-carboxyfluorescein labelled D3 (FAM-D3) in an APPswe/P51A9 transgenic mouse, fluorescence was observed associated with Aβ plaques in brain parenchyma (Fig. 5A). Colocalization of FAM-D3 fluorescence with Aβ aggregates was validated by immunohistochemistry using anti-Aβ antibody (W0-2) based staining on adjacent sections (Fig. 5B and C). Cerebral blood vessels were visualized using an antiglucose transporter GLUT-1 antibody (Fig. 5D). No specific binding of FAM-D3 on cerebral blood vessels was observed (Fig. 5E). An anti-GLUT-1 positive structure was found in the middle of an Aβ deposition, suggesting this plaque was formed around the blood vessel (Fig. 5D and E, white arrows).

3.4. Proteolytic stability of FAM-D3 in comparison to FAM labelled l- enantiomeric peptide

In order to support the interpretation that FAM fluorescence correlates with binding of intact FAM-D3, an ex vivo stability test was performed with brain homogenate. The proteolytic stability of D3 peptide within its amino acid sequence has been reported earlier [7]. In this study, the stability of D3 bound to FAM via an L-Lys linker was tested additionally. As a control, a 12-mer peptide consisting of L-enantiomeric amino acid residues (LP) coupled to FAM was used. FAM-D3 and FAM-LP were incubated with brain homogenate at 37 °C and analysed by thin layer chromatography (TLC) (Fig. 6). Under the same experimental conditions, RF values of FAM-D3 and FAM-LP were different due to their different amino acid sequences. Most of the FAM-LP was degraded in brain homogenate after 2 h, whereas FAM-D3 was resistant to proteolysis within the same time frame.

4. Discussion

The all-D-enantiomeric peptide D3 contains five arginines out of twelve amino acid residues. Three arginines at the N-terminus and two at C-terminus are separated by single spacing amino acids, respectively. This arginine arrangement in peptide backbone may increase its
cellular uptake as studies have shown that increase of the distance between arginine residues enhances the uptake of ARMs[57,58]. The arrangement of arginines at the C-terminus may contribute to the overall similarity of D3 to ARM of HIV-1 Tat, which may lead to the ability to penetrate plasma membranes and the BBB. In order to elucidate this observation, further \textit{in vivo} experiments using tritium labelled ($^{3}$H-D3) and fluorescently labelled (FAM-D3) peptide were carried out.

The systemic administration of $^{3}$H-D3 (i.p.) led to similar concentrations of radioactivity in CSF, plasma and whole brain after 4 h. As $^{3}$H-D3 has been shown to be proteolytically stable, D3 concentrations can be calculated from measured radioactivity and its metabolites can be neglected[7]. Also, temporal distribution of $^{3}$H-D3 in the plasma and brain fitted well with recently reported pharmacokinetic data[7]. The fast appearance and constant presence of D3 in the brain suggests direct penetration into brain parenchyma via the blood brain barrier. It must be noted that, as radioactivity from CSF remained in the ventricles or from the blood in the cerebrovasculature, this may have contributed to the radioactivity detected in the whole brain. But because the CSF space was destroyed during preparation of the brain hemispheres, the amount of remaining CSF in the brain hemisphere was considered negligible. Since we could not detect specific binding of $^{3}$H-D3 to cerebral blood vessels in \textit{in vitro} autoradiography, it is unlikely that radioactivity bound to the vessel walls contributed significantly to the amount detected in the whole brain. Furthermore, the cerebral blood volume decreases dramatically in the absence of any cardiac activity once the mouse is euthanized[59]. Derivation of theoretical concentration in intact brain assuming well preserved cerebral blood and CSF volume was also performed. When maximal blood and CSF compartments (using measured plasma and CSF concentrations) were excluded, there was still about half of the concentration left in the brain. Thus, the $^{3}$H-D3 concentration detected in the brain likely represents its real concentration in the brain parenchyma and the vascular fraction is negligible.

The amount of $^{3}$H-D3 measured up to 4 h post-administration in the brain is 0.14% to 0.21% of the total administered dose, which is clearly above the known bias concentration of 0.10% for BBB permeating agents[60]. Clinically applied CNS drugs consisting of L-polypeptides with proven BBB penetration potential, like Colistin, have CSF/serum ratios in the range of 0.051 to 0.057, or 0.16[61]. In contrast, the CSF/plasma ratio of D3 started at 0.1 and reached 1.2 after 4 h post-administration, suggesting distribution in brain parenchyma. Even if drug entry into the CSF alone is not a proper measure of BBB permeability[62], CSF concentrations of

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Fig. 4. \textit{In vitro} autoradiography of $^{3}$H-D3 on mouse brain cross-sections. (A) Autoradiogram of an APP/PS1 (ARTE 10) transgenic mouse brain section (sagittal) incubated with $^{3}$H-D3. A stronger $^{3}$H-signal correlates to a higher blackening of the image. (B) Thioflavine S staining for amyloid of section A performed after autoradiography. (C) Merged image based on the fluorescence signal after Thioflavine S staining (green) and autoradiography (converted to red). Yellow indicates colocalization of Thioflavine S and $^{3}$H-D3. (D) Merged image with higher magnification. White arrow points to a blood vessel in cerebellum which is not positive for $^{3}$H-D3. Other small spots that only showed Thioflavine S positive stain but were not stained by $^{3}$H-D3 could be either plaques that were only weakly stained by $^{3}$H-D3 or small cerebral blood vessels.

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Fig. 5. Colocalization of FAM-D3 and Aβ in APPswe/PS1ΔE9 transgenic mouse brain sections after eight weeks’ oral administration of FAM-D3. (A and B) Fluorescence images detecting FAM-D3 in cortex with low-magnification (A) and high-magnification (B), respectively. (C) High-magnification of photomicrograph showing adjacent section of B stained with anti-Aβ antibody (WO-2). (D) Microvasculature stained with anti-GLUT-1 antibody on the same section of B. (E) Merged image of B and D. White arrows highlight anti-GLUT-1 positive structure in the center of Aβ aggregate.
drugs are still considered as discriminating factors of drugs and their bioavailability in the CNS, especially for hydrophilic or large molecular weight compounds [63,64].

BBB permeability of D3 was demonstrated and further confirmed by oral administration of FAM labelled D3 in a transgenic AD mouse model. Aβ plaques in the brain were visualized under fluorescent light indicating FAM-D3 entered the brain parenchyma and bound to Aβ plaques. Together with the proteolytic stability test, results showed that D3 was orally active. D3 is selected to specifically bind to Aβ(1–42) plaques, which is the major component of parenchymal Aβ plaques, whereas the major Aβ species in cerebral amyloid angiopathy (CAA) is Aβ(1–40) (however, upon aging, more and more Aβ(1–42) can be found in CAA) [65,66]. No specific binding of FAM-D3 on cerebral blood vessels was found, which is in accordance with our observations during the study when incubating 3H-D3 with mouse brain sections. We also detected blood vessels in close proximity to Aβ plaques (Fig. 5E), supporting the hypothesis that every plaque seems to be associated with a vessel [67,68].

In our study, BBB penetration of D3 was quantified by measuring its brain concentration after i.p. administration (using 3H-D3) and visualized through its binding to Aβ plaques inside the brain (using FAM-D3 and FAM-LP). However, all those methods have their restrictions and thus needed to be validated by each other: D3 concentration in brain was calculated from radioactivity of tritium labelled D3, assuming that D3 did not bind to the CNS vasculature, which otherwise would have lead us to misinterpreted brain levels (Fig. 4). In addition, labelling with a bulky, hydrophilic FAM lysine molecule adding one extra negative charge to D3 could have an influence on its BBB penetration and Aβ binding. Thus combination of experiments in Figs. 2 and 4 demonstrated that 3H-D3, which preserves the original physical and chemical properties of D3 to the fullest extent, could pass the BBB and bind Aβ plaques.

Re-analysis and interpretation of the data reported previously [7] and in combination with Figs. 2 and 3, suggests that high concentrations of D3 in plasma do not directly translate into similarly high D3 concentrations either in brain or in CSF. After i.p. administration the plasma concentration of D3 rises rapidly to reach its maximum before dropping away within the first hours. In contrast, brain concentrations do not follow the same pattern but rise until certain and very stable levels are reached. Similar brain levels could previously be found after i.v. and even after p.o. administration despite huge differences by the order of several magnitudes in the initial plasma levels after i.v., i.p. and p.o. administration. Those obviously constant brain levels may suggest that the responsible transport mechanism from plasma into the CNS is kinetically limited and that elevating the plasma concentration dose not directly lead to a proportional increase of brain concentration. This is in perfect agreement with a previous report on the permeability of D3 in an in vitro BBB model [43]. On a co-culture of rat brain microvascular endothelial cells and rat astrocytes in a transwell filter system, D3 passed through the in vitro BBB model and showed a partially saturated apical-to-basolateral (blood to brain) transport pattern, whereas another D-peptide (qshyrhispaq) used as a control containing only one arginine did not pass the in vitro BBB model. This pattern was further investigated and it was suggested that D3 might be transported via adsorptive-mediated transcytosis [43,69].

Arginine-rich HIV-Tat-like peptides were suggested to bind negatively-charged cell membrane embedded molecules such as heparan sulfate and sialic acid [70–72]. If the cell membrane-permeating property of Tat is independent of cell surface receptor proteins, it is likely that this process is rather dependent on the overall charge density, than on the presence of any specific sequence or secondary structure elements. Thus, the configuration of peptides (chirality) should not be relevant for cell membrane penetration. In the study of Tünnemann et al., D-enantiomeric arginine-rich peptides showed improved ability to cross the cell membrane as compared to their L-enantiomers, which was explained as a result of difference in proteolytic stability [73].

The observed BBB permeability of D3 in the hereby presented in vivo study is in agreement with this concept. Tünnemann et al. also investigated the cell penetration ability of oligo-arginines (with 5 to 12 arginine residues) coupled directly to fluorescein or 5,6-carboxytetramethylrhodamine (TAMRA). Their results show that the transduction ability of arginines increase with the number of consecutive residues and the best performance associated with a tolerable toxicity is achieved with 9 and 10 arginines [73]. Other studies reported that not only linear peptides but also branched-chain peptides show efficient transduction with an optimum number of approximately 8 arginines [74,75]. In this study, D3 achieved the in vivo BBB permeability through 5 arginine residues almost equally distributed over the whole molecule, which suggests a cation (guanidinium) dependent and sequence/structure related electrostatic interaction of D3. This special feature not only enables protease-resistant D3 to interact with Aβ, but also has advantage over the strategy to add extra arginine-rich peptides to achieve BBB permeability, as more arginine residues are usually accompanied with increased toxicity and production costs [73,76].

Another study showed that arginine-rich peptides are able to directly penetrate the plasma membrane independent of endocytosis [77]. The formation of nonselective pores was also excluded, because simultaneously added fluorophores were not taken up together with the arginine-rich peptides. BBB penetration of D3 might follow a similar mechanism as HIV-Tat assuming the limited transport pattern mentioned above. In fact, no complex transport mechanism is necessary to explain the distribution pattern of D3 in the body. It could be described as a rapid absorption phase from peritoneal cavity into systemic circulation followed by slower passive entry into intracellular compartments of brain and other organs, after which the proteolytically stable D3 remains equilibrated throughout the body. Remarkably, our results showed a relatively constant brain/CSF ratio over all time points, which also indicated a distribution equilibrium of D3 between brain and CSF. The most straightforward explanation would be that this equilibrium, as well as differences in brain/plasma and CSF/plasma ratios may simply reflect reduced distribution to non-aqueous compartments in brain that do not exist in CSF. In addition, this constant brain/CSF ratio might also provide a possibility to monitor D3 concentrations in brain, because CSF sampling can be performed several times or even continuously in living animals.

Taken together, our results strongly suggest that D3 penetrates the blood brain barrier and specifically binds to Aβ plaques after systemic administration. Being orally active, D3 might be a promising drug...
candidate for therapeutic intervention in Alzheimer's disease. Additionally, being a middle-sized peptide of synthetic origin, D3 allows easy chemical modifications e.g. radiolabelling for early diagnosis of the disease.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jbamen.2016.07.002.

Conflict of interest

There is no conflict of interest regarding this manuscript.

Author contributions

Conceived and designed the experiments: NJ, DF, TVG, IK, NJS, KJL, DW, AW

Performed the experiments: NJ, ES, TVG, IK

Analysed the data: NJ, TVG, IK, DW, AW

Wrote the paper: NJ, TVG, IK, KJL, DW, AW

Transparency document

The Transparency document associated with this article can be found, in the online version.

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