Surprisingly High Stability of the Amyloid β Peptide Targeting All-D-Enantiomeric Peptide D3 in Media Simulating the Route of Orally Administered Drugs

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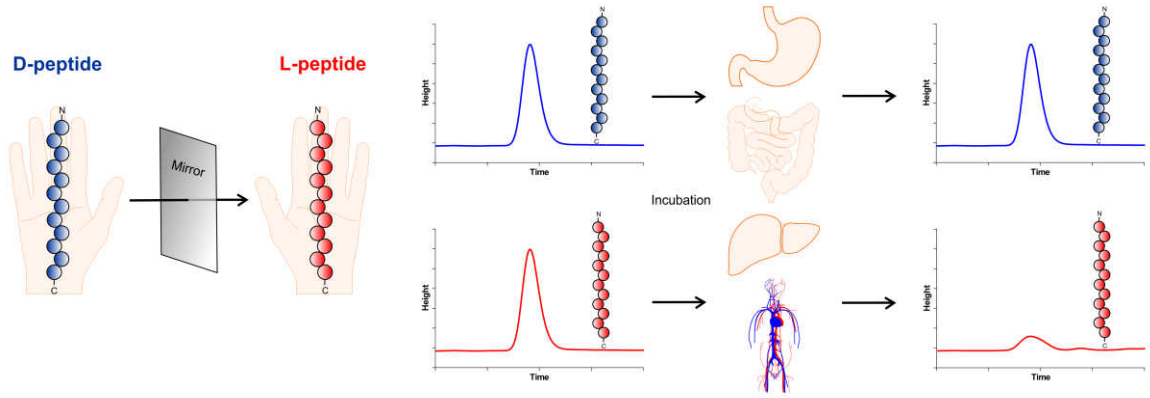

Abstract

The aggregation of the amyloid β protein (Aβ) plays an important role in the pathology of Alzheimer's disease. Previously, we have developed the all-D-enantiomeric peptide D3, which is able to eliminate neurotoxic Aβ oligomers *in vitro* and improve cognition in a transgenic Alzheimer's disease mouse model *in vivo* even after oral administration. D-Peptides are expected to be more resistant against enzymatic proteolysis compared to their L-enantiomeric equivalents, and indeed, a pharmacokinetic study with tritiated D3 revealed the oral bioavailability to be about 58%. To further investigate the underlying properties, we examined the stability of D3 in comparison to its corresponding all-L-enantiomeric mirror image L-D3 in media simulating the gastrointestinal tract, blood and liver. Potential metabolization was followed by reversed-phase high-performance liquid chromatography. In simulated gastric fluid, D3 remained almost completely stable (89%) within 24 h, while 70% of L-D3 was degraded within the same time period. Notably, in simulated intestinal fluid, D3 also remained stable (96%) for 24 h, whereas L-D3 was completely metabolized within seconds. In human plasma and human liver microsomes L-D3 was metabolized several hundred times faster than D3. The remarkably high stability may explain the high oral

bioavailability seen in previous studies allowing oral administration of the drug candidate. Thus, all-D-enantiomeric peptides may represent a promising new compound class for drug development.

Keywords

Alzheimer's disease; Amyloid beta protein; All-D-enantiomeric peptide; Oral administration; Oral stability; Metabolism

1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and represents 60 to 80% (Alzheimer's Association, 2016) of the currently 47 million dementia cases worldwide with continuously increasing numbers of patients (Prince et al., 2016). The aggregation of the neurotoxic amyloid β peptide (Aβ) is thought to initiate AD pathology leading to characteristic neuritic extracellular amyloid plaques, tau aggregates and loss of neurons in the brain (Hardy and Higgins, 1992; Selkoe and Hardy, 2016).

Despite intensive efforts in drug development, no preventive or curative treatment has been achieved yet (Huang and Mucke, 2012). Besides low molecular weight chemical entities, peptides consisting of L-enantiomeric amino acid residues gain increasing interest (Funke and Willbold, 2012; Sun et al., 2012). Although they offer a variety of favorable qualities like high biological activity and specificity as well as low toxicity (Lien and Lowman, 2003; Sun et al., 2012), they also have disadvantages, like almost no oral bioavailability and rapid proteolytic degradation and clearance (Adessi and Soto, 2002; Gomez-Orellana, 2005; Hamman et al., 2005; Sato et al., 2006). To overcome these disadvantages, several promising strategies were developed. One of these is the replacement of L- against D-enantiomeric amino acid residues. Previously, it has been shown that D-peptides are proteolytically more stable than L-peptides (Elmquist and Langel, 2003; Findeis et al., 1999; Miller et al., 1995; Poduslo et al., 1998; Soto et al., 1996; Tugyi et al., 2005; Wang et al., 2015; Werle and Bernkop-Schnurch, 2006) because proteases are stereoselective for L-amino acid residues (Van Regenmortel and Muller, 1998).

We have identified the all-D-enantiomeric peptide D3 by mirror image phage display against monomeric and small oligomeric Aβ(1-42) (Funke and Willbold, 2009; Schumacher et al., 1996; Wiesehan and Willbold, 2003). This lead compound consists of 12 amino acid residues each in D-enantiomeric configuration. *In vitro* assays revealed that D3 specifically eliminates Aβ oligomers (Brener et al., 2015; Funke and Willbold, 2012), which are supposed to be the most toxic Aβ species (Benilova et al., 2012; DaRocha-Souto et al., 2011; Lambert et al., 1998; Walsh et al., 2002). In studies with AD transgenic mice, D3 reduced the Aβ plaque load as well as cerebral inflammation and showed an improvement in cognition (van Groen et al., 2012; van Groen et al., 2013; van Groen et al., 2008) even after oral administration (Funke et al., 2010). A pharmacokinetic study with the tritiated peptide revealed that D3 is characterized by high oral bioavailability, long blood circulation (Jiang et al., 2015) and efficient blood brain barrier permeability (Liu et al., 2010). The high oral bioavailability is based on an efficient intestinal absorption and probably also on a high resistance against metabolization during the gastrointestinal passage.

In the current study, we investigated the resistance of the all-D-enantiomeric peptide D3 against metabolization *in vitro* in media simulating the route of orally administered drugs, like the gastrointestinal tract, blood and liver, in comparison with the corresponding all-L-enantiomeric mirror image L-D3. We followed metabolization by reversed-phase high-performance liquid chromatography (RP-HPLC).

2 Material & methods

2.1 Peptides

The D-peptide D3 (sequence: rprtrlhthrnr) consists of 12 amino acid residues each in D-configuration with its C-terminus being amidated. The mirror image of D3, L-D3, has the same amino acid sequence but with all amino acid residues in L-configuration. D3 and L-D3 have a molecular weight of approximately 1.6 kDa. The peptides were obtained from Peptides & Elephants (Potsdam, Germany).

2.2 Media simulating the gastrointestinal tract, blood and liver

Preparation of simulated gastric and intestinal fluid was performed according to the European Pharmacopoeia 7.0. Simulated gastric fluid (SGF) was prepared by acidifying distillated water with 80 mM hydrochloric acid and adding 3.2 mg/ml pepsin from porcine stomach (EC 3.4.23.1; Carl Roth, Karlsruhe, Germany; CAS: 9001-75-6, 0230.1, ≥0.5 units/mg Ph. Eur.). The final pH was 1. Simulated intestinal fluid (SIF) was prepared by dissolving 0.2 M potassium dihydrogen phosphate in distilled water, adjusting the pH to 6.8 with 15.4 mM sodium hydroxide and adding 10 mg/ml pancreas powder from porcine pancreas (Sigma-Aldrich, St. Louis, USA, CAS: 8049-47-6, P7545, 8x USP). SGF sine pepsin (SGFsp) and SIF sine pancreatin (SIFsp) were prepared like SGF and SIF but without enzymes.

Plasma samples were obtained from human blood of a volunteer female donor. Blood was taken via a venous cannula. K_3-EDTA served as anti-coagulant. The blood sample was centrifuged at 3000*g* at 4 °C for 10 min to obtain the cell free plasma.

Pooled human liver microsomes were purchased from Sigma-Aldrich (St. Louis, USA; M0567, Lot: SLBN7300V). The liver microsomes were diluted in a NADPH regenerating system (NRS) to a final concentration of 4.8 mg/ml (20-fold excess regarding peptide concentration in mg/ml). NADPH serves as reducing agent for the enzyme family cytochrome P450 (CYP) and is therefore an essential factor for their activity. The NRS consisted of 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase and 3.3 mM magnesium chloride in 100 mM potassium phosphate buffer (pH 7.4).

2.3 Incubation of D3 and L-D3 in SGF, SIF, human blood plasma and human liver microsomes

150 µM D3 or L-D3 was incubated in SGF, SIF, human plasma and human liver microsomes in triplicate at 37 °C with slight shaking for different time periods. To prevent microbial contaminations in longterm plasma incubations, 0.1% sodium azide was added to the

solutions. The incubation was stopped after different periods of time by precipitating the proteins with 3% trichloroacetic acid (w/v) under vortexing. The peptides were extracted from the media by centrifugation at 14000*g* at 4 °C for 5 min. The supernatant containing the peptides was snap-frozen in liquid nitrogen immediately and stored at -80 °C for further analysis. As reference for quantification, the peptides were transferred into the media and the incubation was stopped immediately by precipitating the proteins. Precipitated media without peptides served as controls.

2.4 Quantification of unmetabolized D3 and L-D3 by reversed-phase high-performance liquid chromatography (RP-HPLC)

Potential peptide metabolization was followed by RP-HPLC. The samples were injected on a C18 column (Agilent Technologies, Santa Clara, USA; ZORBAX 300SB-C18 5 µm, 4.6 x 250 mm). Mobile phases were acetonitrile with 0.15% trifluoroacetic acid (TFA) (v/v) (solvent A) and water with 0.15% TFA (v/v) (solvent B). The samples were measured isocratically at 10% solvent A at 25 °C with a flow rate of 1 ml/min. This run conditions allowed us to detect the smallest chemical changes and differentiate even between amidated and deamidated peptide forms, which usually elute simultaneously. Namely, D3 and L-D3 can possibly be deamidated at two sites, the C-terminus and the asparagine at position seven. Chromatograms were recorded and analyzed with the Agilent software ChemStation (G2175BA; B03.01). The peptides' peak area after direct extraction from the media was set to 100%. Peak areas of the unmetabolized peptides after different incubation times were normalized to this. Normalized peak areas of each measurement in triplicate were averaged. Data are presented as mean ± SD.

2.5 Confirmation of peptide deamidation by electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-QTOF-MS)

To confirm the identity of a one-fold deamidated D3 or L-D3 metabolite observed during RP-HPLC measurements mentioned above, we collected the respective substance peak during the run. The sample was freeze-dried and subsequently dissolved and diluted in a solution composed of 85% water, 15% acetonitrile and 0.1% formic acid (v/v/v). ESI-QTOF-MS infusion experiments of the samples were performed on an Agilent 6250 Accurate QTOF-MS (Agilent Technologies, Santa Clara, USA) in the positive mode. For the calibration of the MS the G1969-8500 QTOF standard calibration mix from Agilent was used. While the amidated peptide has a monoisotopic mass of 1597.914 Da and would be detected in the charge of 3 with m/z of 533.64 Da, the one-fold deamidated peptide has a monoisotopic mass of 1598.898 Da and was detected in the charge of 3 with m/z of 533.98 Da.

2.6 Recording of a chromatographic metabolite profile of L-D3 in SIF

Additionally, a chromatographic metabolite profile of L-D3 in SIF was recorded by RP-HPLC. The samples were injected on a C18 column (Agilent Technologies, Santa Clara, USA; ZORBAX 300SB-C18 5 µm, 4.6 x 250 mm). Mobile phases were acetonitrile with 0.15% trifluoroacetic acid (TFA) (v/v) (solvent A) and water with 0.15% TFA (v/v) (solvent B). The samples were measured with a gradient from 0 to 30% solvent A in 30 min at 25 °C with a flow rate of 1 ml/min. Chromatograms were recorded and analyzed with the Agilent software ChemStation (G2175BA; B03.01).

3 Results

To determine whether the D-peptide D3 is more resistant against metabolization in the gastrointestinal tract, blood and liver than its mirror image L-D3, both peptides were incubated in simulated gastric and intestinal fluid (SGF and SIF) as well as in human plasma and human liver microsomes. The resistance against metabolization was quantified by RP-HPLC.

3.1 Simulated gastric fluid

In SGF, the resistance of D3 and L-D3 against degradation was monitored for up to 24 h. While the D-peptide remained almost completely stable (89 ± 1%) within 24 h (Fig. 1), 70 ± 3% of L-D3 was metabolized during this time. A proportion of the metabolization of L-D3 accounts for deamidation at one of the two possible deamidation sites within the peptide. Up to 23 ± 2% regarding the initial L-D3 amount was one-fold deamidated within 24 h. Deamidation of L-D3 at one site, which results in a mass shift of +0.98 Da regarding D3's mass, was additionally confirmed by ESI-QTOF-MS (data not shown). In contrast, D3 was not deamidated in SGF.

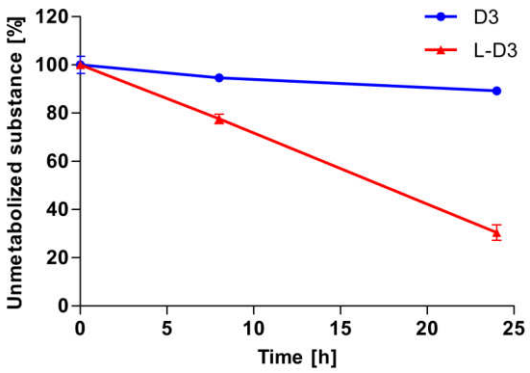

Fig. 1. Stability of D3 and L-D3 in simulated gastric fluid. D3 and L-D3 were incubated in simulated gastric fluid. D3 remained almost completely stable (89 ± 1%) for 24 h, whereas 70 ± 3% of L-D3 was metabolized within the same time. Peak areas of the unmetabolized peptides after different incubation times were normalized to the peptides' peak areas after direct extraction from SGF. Data are presented as mean ± SD (n = 3).

3.2 Simulated intestinal fluid

The resistance of D3 and L-D3 against metabolization was also investigated in SIF for up to 24 h. D3 remained stable during this time (96 ± 4%) (Fig. 2A). In contrast, L-D3 was completely metabolized within a few seconds. To confirm the immediate metabolization of L-D3, a chromatographic metabolite profile of L-D3's metabolites after incubation in SIF for a few seconds was recorded and compared to the profile of L-D3 incubated in SIF sine pancreatin (SIFsp) (Fig. 2B). No L-D3 but several metabolites could be detected.

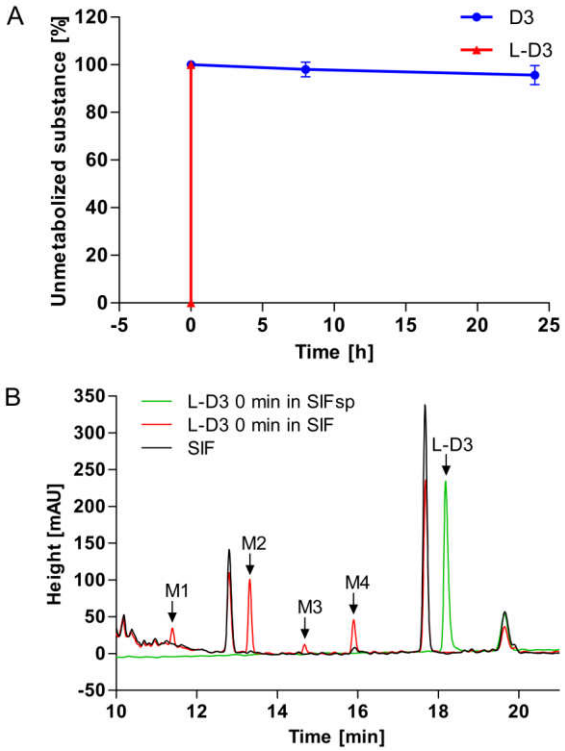

Fig. 2. Stability of D3 and L-D3 in simulated intestinal fluid. D3 and L-D3 were incubated in simulated intestinal fluid (SIF). **(A)** D3 remained stable for 24 h (96 ± 4%), whereas L-D3 could not be detected already after a few seconds of incubation. Peak areas of the unmetabolized peptides after different incubation times were normalized to the peptides' peak areas after direct extraction from SIF. Data are presented as mean ± SD (n = 3). **(B)** A chromatographic metabolite profile of L-D3 was recorded after incubation in SIF for a few seconds and was compared to the profile of L-D3 in SIF sine pancreas powder (SIFsp). L-D3 could not be detected, but several metabolites occurred (M1-4).

3.3 Human plasma

In human plasma, the stability of D3 was monitored over a time period of 20 days, whereas the degradation of L-D3 was followed for 2 h only due to faster degradation. L-D3 was metabolized > 500 times faster than D3 (Fig. 3). In detail, 74 ± 4% of D3 was degraded within 20 days, whereas 81 ± 1% of L-D3 was already degraded within 1 h.

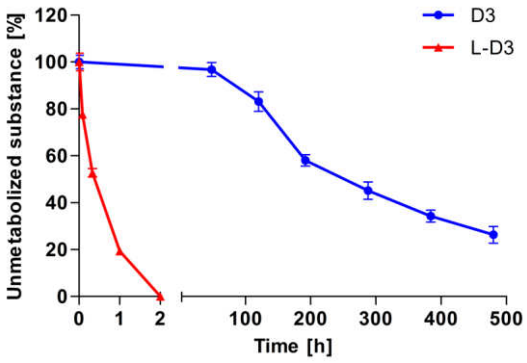

Fig. 3. Stability of D3 and L-D3 in human plasma. D3 and L-D3 were incubated in human plasma. 74 ± 4% of D3 was degraded within 20 days, whereas 81 ± 1% of L-D3 was already degraded within 1 h. Peak areas of the unmetabolized peptides after different incubation times were normalized to the peptides' peak areas after direct extraction from plasma. Data are presented as mean ± SD (n = 3).

3.4 Human liver microsomes

Furthermore, metabolization of D3 and L-D3 was examined in human liver microsomes for 24 h and 30 min, respectively. L-D3 was almost completely degraded within 30 min (96 ± 0.3%), whereas only 30 ± 3% of D3 was metabolized within 24 h (Fig. 4A). To prove microsomal long-term activity, we incubated microsomes without peptides for up to 24 h and examined their activity by their ability to degrade L-D3, which was added after 8 and 24 h. The results show that the microsomes remained active during the whole experiment (Fig. 4B).

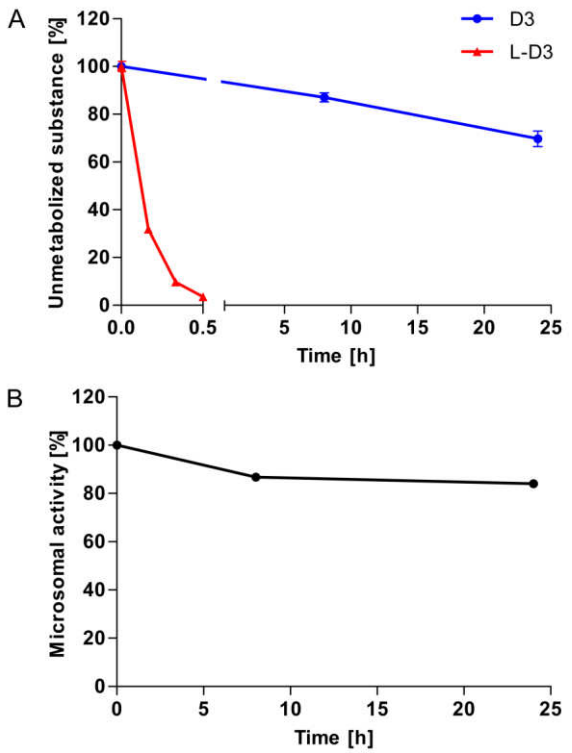

Fig. 4. Stability of D3 and L-D3 in human liver microsomes. D3 and L-D3 were incubated in human liver microsomes. **(A)** While 30 ± 3% of the the D-peptide was metabolized within 24 h, the L-peptide was almost completely degraded within 30 min (96 ± 0.3%). **(B)** Microsomal activity with the NADPH regenerating system was examined for 24 h. The microsomes remained active during the whole experiment. Peak areas of the unmetabolized peptides after different incubation times were normalized to the peptides' peak areas after direct extraction from microsomes. Data are presented as mean ± SD (n = 3).

4 Discussion

Oral application is in general the preferred administration route of drugs because of low invasiveness, low risk of microbial contamination, good patient compliance, cost-effectiveness and flexibility in the design of the dosage form. Especially for patients with Alzheimer's disease, it is important to have a feasible administration form for long-term application. Peptide-based drugs gain considerable interest due to their high biological activity and specificity as well as low toxicity (Lien and Lowman, 2003; Sun et al., 2012). However, a critical drawback of orally administered pharmaceuticals, especially in the case of peptide-based drugs, is their instability in the gastrointestinal tract, blood and liver (Adessi and Soto, 2002; Gomez-Orellana, 2005; Hamman et al., 2005; Sato et al., 2006).Thus, it is not surprising that it has been tried to increase the stability of peptides by the conversion of single or all L-amino acid residues against D-amino acid residues to promote a distinct enhancement of the metabolic stability in several different body fluids and tissues (Elmquist and Langel, 2003; Miller et al., 1995; Tugyi et al., 2005; Wang et al., 2015; Werle and Bernkop-Schnurch, 2006). This strategy was also applied for some D-peptides targeting Aβ (Findeis et al., 1999; Kumar and Sim, 2014; Poduslo et al., 1998; Soto et al., 1996).

Previously, we have developed the all-D-enantiomeric peptide D3, which specifically eliminates neurotoxic Aβ oligomers *in vitro* (Brener et al., 2015; Funke and Willbold, 2012), has a high oral bioavailability (Jiang et al., 2015) and is therapeutically active *in vivo* (van Groen et al., 2012; van Groen et al., 2013; van Groen et al., 2008) even after oral administration (Funke et al., 2010).

In the present study, we have analyzed the stability of D3 in comparison to its mirror image L-D3 *in vitro* in media simulating the route of orally administered drugs, like the gastrointestinal tract, blood and liver. To our knowledge, this is the first time such a rigorous comparison between two enantiomers of one amino acid sequence has been done. Especially in the gastrointestinal tract it has not been published in that detail yet.

In our study, D3 was incubated in simulated gastric and intestinal fluid for 24 h. The typical residence time of particles in the stomach is 15 min to 13 h, and in the small intestine it is 1 to 3 h depending on the particle size and if the organ is in a fasted or fed state (Dressman et al., 1998). Hence, monitoring the degradation for 24 h was more than sufficient to get an impression of D3's stability in the gastrointestinal tract. The terminal half-life of orally administered D3 in plasma has been shown to be >24 h in a pharmacokinetic study (Jiang et al., 2015). We incubated D3 in plasma even for 20 days. The actual residence time in the liver cannot be predicted because the peptide may pass through the liver several times

during the enterohepatic circulation. We decided to perform our experiments for a maximum of 24 h under monitoring of the activity of the NADPH regenerating system.

Here, we demonstrate that D3 is substantially more resistant against metabolization in simulated gastric and intestinal fluid, human plasma and human liver microsomes than its L-enantiomeric mirror image L-D3. In SGF, D3 remained almost completely stable for 24 h, whereas 70% of L-D3 was degraded within the same time. In SIF, D3 also remained stable for 24 h, while L-D3 was degraded entirely after a few seconds. In blood plasma and liver microsomes, the L-peptide was metabolized several hundred times faster than the D-peptide.

L-D3 has several potential cleavage sites for proteases contained in the gastrointestinal fluid and blood like pepsin, trypsin, chymotrypsin (Keil, 1992) and different carboxypeptidases (Christianson and Lipscomb, 1989; Folk, 1956; Lipscomb, 1970). These cleavage sites are obviously less recognized by proteases within the D-peptide. The proteases seem to be very specific for L-amino acid peptide bonds. Thus, it was not surprising that the L-peptide was degraded many times faster than the D-peptide.

The liver plays a major role in drug metabolism, especially on the first-pass metabolism after oral drug administration. Drugs entering the liver undergo phase I and phase II metabolic biotransformation. During phase I metabolism reactive and polar groups are introduced into the substrate (Guengerich, 2001). During subsequent phase II metabolism the substrate is conjugated, which favors the compound's excretion and inactivation (Jancova et al., 2010). Metabolites modified by phase I reactions are generally more pharmacologically active than phase II metabolites (U.S. Food and Drug Administration, 2008). To this end, it is crucial to investigate the drug's phase I metabolism. In this study, we used liver microsomes containing phase I enzymes, which contain the protein family cytochrome P450 (CYP) (Lu, 1976). CYP enzymes are mainly monooxygenases, which catalyze the substrate's hydroxylation (Guengerich, 2001; Lu, 1976). D3 clearly showed higher resistance against enzymatic modifications in the liver microsomes than its L-enantiomeric mirror image.

In conclusion, the high resistance against metabolization in the gastrointestinal tract guarantees high availability for absorption by the intestine and lets us assume that D3's high oral bioavailability can be explained based on its ability to pass through the gastrointestinal tract upon oral administration entirely and unmodified without protective formulation. Additionally, the high resistance against metabolization in the blood and liver may contribute to the high bioavailability and long terminal half-life of D3 in the blood as observed previously (Jiang et al., 2015). D3 has been shown to improve cognition in AD mice after oral treatment for eight weeks with roughly 30 mg/kg per day (Funke et al., 2010), which is, according to the hereby presented results, certainly attributed to its ability to pass through the gastrointestinal

tract unmetabolized as well as to its high blood brain barrier permeability with a brain-plasma distribution ratio of about 0.8 (Jiang et al., 2015). Thus, all-D-enantiomeric peptides may represent a promising new class of compounds for drug development.

Declaration of interest

The authors declare no competing financial interests.

Submission declaration and verification

The authors guarantee that the manuscript describes original work, is not under consideration for publication concurrently and has not been published elsewhere in any medium including electronic journals and computer databases of a public nature. All authors approved the manuscript and this submission.

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