Oral absorption enhancement of the amyloid-β oligomer eliminating compound RD2 by conjugation with folic acid

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Abstract

Amyloid-β (Aβ) plays a central role in the development and progression of Alzheimer's disease (AD) with Aβ oligomers representing the most toxic species. The all-D-enantiomeric peptide RD2, which recently successfully completed clinical phase I, specifically eliminates Aβ oligomers *in vitro* as well as *in vivo* and improves cognitive deficits in various transgenic AD mouse models even after oral administration. To further enhance the oral absorption of RD2, folic acid has been conjugated to the D-peptide promoting an endocytosis-mediated uptake via a folate receptor located in the intestine. Two different conjugation strategies were selected to obtain prodrugs with folic acid being cleaved after intestinal absorption releasing unmodified RD2 in order to enable RD2's unaltered systemic efficacy. Both conjugates remained stable in simulated gastrointestinal fluids. But only one of them was suitable as prodrug as it was cleaved to RD2 *in vitro* in human blood plasma and liver microsomes and *in vivo* in mice after intravenous injection leading to a systemic release of RD2. Furthermore, the conjugate's permeability *in vitro* and after oral administration in mice was strongly enhanced compared to unconjugated RD2 demonstrating the prodrug's functionality. However, the conjugate seemed to have impaired the mice's wellbeing shortly after oral administration possibly resulting from strain-specific hypersensitivity to folic acid. Nevertheless, we assume that the prodrug is actually non-toxic, especially in lower concentrations as verified by a cell viability test. Furthermore, lower dosages can be applied with unaltered efficacy due to its enhanced oral absorption.

Keywords: prodrug, folic acid conjugate, intestinal absorption, all-D-enantiomeric peptide, Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder leading to dementia (Kodama et al., 2010; Reitz and Mayeux, 2014) that is still in urgent need of a successful therapy (Karran et al., 2011). The self-assembly of the amyloid-β protein (Aβ) misfolding is crucial for the development and progression of the disease (Masters et al., 1985; Selkoe and Hardy, 2016), where especially Aβ oligomers seem to have neurotoxic properties (Benilova et al., 2012; DaRocha-Souto et al., 2011; Lambert et al., 1998; Walsh et al., 2002). We developed the very promising therapeutic D-enantiomeric peptide RD2, which directly eliminates Aβ oligomers *in vitro* and *in vivo* (Schemmert et al., 2019b; van Groen et al., 2017), improves cognitive and motor deficits of AD transgenic mice (Kutzsche et al., 2017; Schemmert et al., 2019a; Schemmert et al., 2019b; van Groen et al., 2017) and demonstrated safety and tolerability in humans in a phase I clinical trial (Kutzsche et al., 2020). Oral drug administration is not only the most common administration form but also the administration form with the strongest drug metabolization and least bioavailability. The drug candidate therefore optimally needs to have a high stability in the gastrointestinal tract, an efficient intestinal absorption and a minor systemic metabolization. For RD2, it could be shown that it has an exceptional high stability in media simulating the oral administration form, has an oral bioavailability with a terminal half-life of approx. 60 h (Leithold et al., 2016) and improves cognitive and motor deficits of different AD transgenic mice after oral treatment (Kutzsche et al., 2017; Schemmert et al., 2019a; Schemmert et al., 2019b).

We investigated, whether a further increase of the intestinal absorption could potentially increase the efficacy of the drug at the same or even lower dosage. The current study describes the attempt to improve RD2's intestinal absorption by covalent coupling to folic acid. In the intestinal epithelium, various folic acid receptors, such as the proton-coupled folate transporter (PCFT), the folate transporter 1 (RFC) and the folate receptor α (FRα), are expressed (Parker et al., 2005; Zhao et al., 2009), but only the FRα mediates uptake of the ligand into the cell by endocytosis (Kamen and Capdevila, 1986; Leamon and Low, 1991; Zhao et al., 2011) enabling the absorption of larger molecules such as peptides. The FRα is expressed specifically in the colon and rectum (Holm et al., 1994; Parker et al., 2005) and could thus favor systemic uptake.

In this work, two different folic acid RD2 conjugates have been developed to increase the intestinal absorption of RD2. While the pteroate moiety of folic acid binds inside the FRα binding pocket, the glutamate moiety sticks out of the pocket entrance allowing drug conjugation without affecting folic acid binding (Chen et al., 2013). Therefore, folic acid was coupled to RD2 via its glutamate moiety. The conjugates were designed to be ideally stable in the gastrointestinal tract and to release folic acid from RD2 only in the intestinal epithelial cells

or in the blood and liver in order to ensure RD2 retains its unaltered efficacy. The two folic acid RD2 conjugates were designed with different stable bonds between ligand and drug.

First, it was examined whether any of the conjugates has a cytotoxic potential and whether the conjugates also reduce $A\beta_{1-42}$-induced cytotoxicity as unconjugated RD2 does. FA(p)RD2's and FA(t)RD2's metabolization to the unconjugated drug has been studied in simulated gastric and intestinal fluids, human blood plasma and human liver microsomes *in vitro* as well as in mouse plasma samples taken 60 min after intravenous injection. Furthermore, the intestinal permeability of RD2 and FA(t)RD2 has been compared in a Caco-2 cell model and the oral absorption of FA(t)RD2 has been investigated in mice.

2. Material & Methods

2.1 Peptides

RD2 (sequence: ptlhthnrrrrr; CBL Patras, Patras, Greece) consists of 12 D-enantiomeric amino acid residues with its C-terminus being amidated. RD2 has a molecular mass of 1598.8 Da. The internal standard for RD2 (sequence: ptvhthnrrrrr; peptides & elephant, Potsdam, Germany) has the same sequence as RD2 with an amino acid substitution at position 3 - leucine versus valine. The peptide has a molecular weight of 1586.0 Da. Two different folic acid RD2 conjugates were synthesized by peptides & elephants (Potsdam, Germany). For FA(t)RD2, the α- or γ-carboxyl group of folic acid was coupled via a glycine linker to the hydroxyl group of threonine at position 2 of the RD2 sequence (Fig. 1). FA(t)RD2 has a molecular mass of 2079.08 Da (Fig. S1 A) and a purity of 91.2% (Fig. S1 C). For FA(p)RD2, the α- or γ-carboxyl group of the folic acid was coupled via the secondary amine of the N-terminal proline of RD2 (Fig. 1). The peptide has a molecular weight of 2021.05 Da (Fig. S1 B) and a purity of 98.3% (Fig. S1 D).

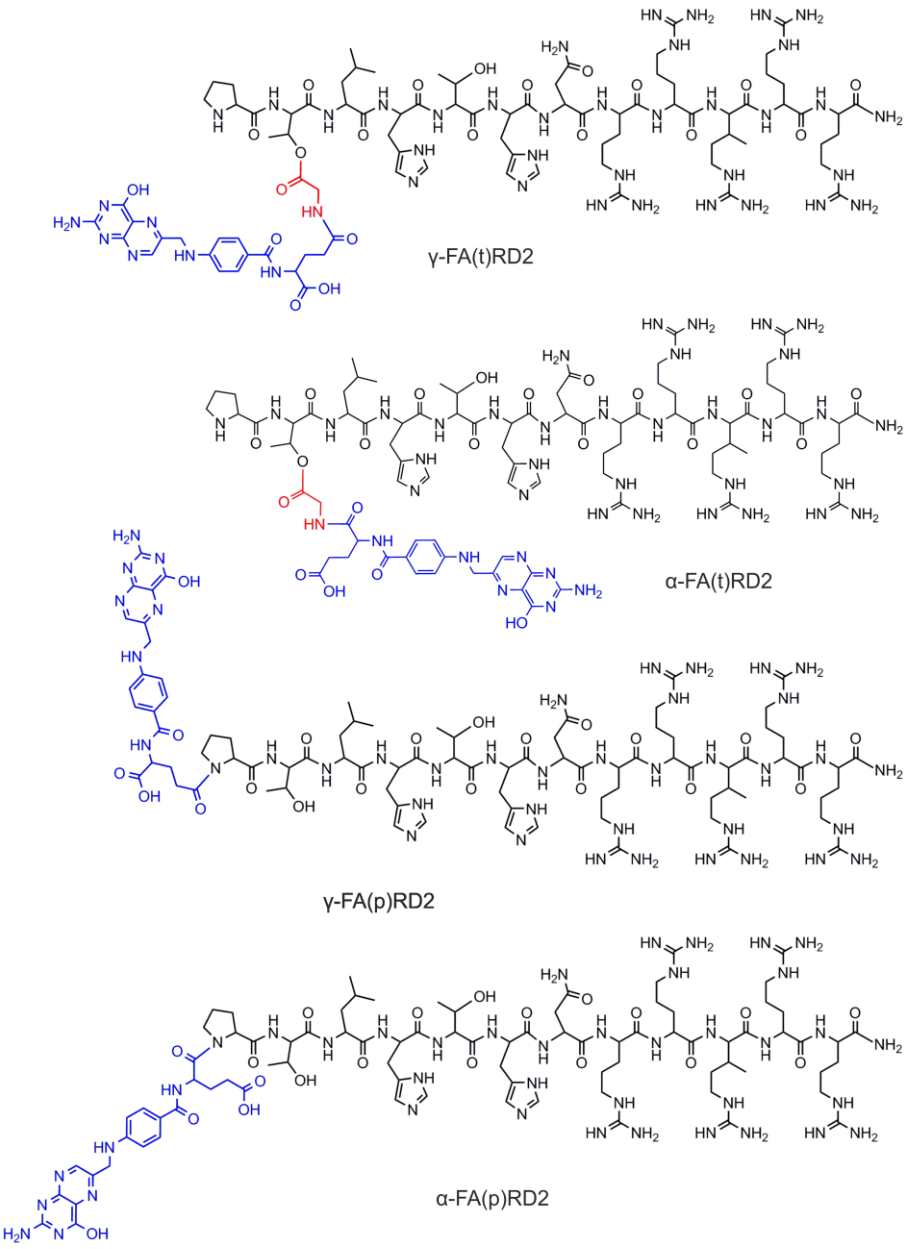

Fig. 1: Structural formulas of both FA(t)RD2 and FA(p)RD2 isomers. RD2: black; folic acid: blue; glycine linker: red.

2.2 Cell viability assay

To investigate potential cytotoxicity of FA(t)RD2, FA(p)RD2 and to compare their influence on the cytotoxic potential of $A\beta_{1\text{-}42}$ to the efficacy of the lead compound RD2, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability test was performed.

Rat pheochromocytoma cells (PC12) (Leibniz Institute DSMZ, Braunschweig, Germany) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 5% horse serum and 1% penicillin-streptomycin. 10,000 cells per well were seeded on collagen A-coated 96-well plates (Gibco, Life Technology, Carlsberg, USA) in a volume of 100

µL per well and incubated in a 95% humidified atmosphere with 5% CO_2 at 37 °C for 24 h. The samples were freshly prepared on the day of the study according to the following scheme: FA(t)RD2, FA(p)RD2 or RD2 was dissolved in 10 mM sodium phosphate buffer (pH 7.4) to 255 µM. 51 µM $A\beta_{1-42}$ was incubated at 21 °C with gentle shaking for 4.5 h in 10 mM sodium phosphate buffer (pH 7.4) to obtain cytotoxic Aβ oligomers. Subsequently, 255 µM FA(t)RD2, FA(p)RD2 or RD2 was added and incubated for further 40 min without shaking. Incubated $A\beta_{1-42}$ and buffer without addition of peptides served as positive and negative controls. 5 µL of the samples was added to the cells in quintuple and incubated at 37 °C and 5% CO_2 for 24 h. The final concentrations were 5 µM peptides and 1 µM $A\beta_{1-42}$. The MTT cell viability test was performed according to the manufacturer's instructions (Cell Proliferation Kit I (MTT), Roche, Basel, Switzerland). The experiment was carried out in triplicates for FA(t)RD2 and RD2 and once for FA(p)RD2. The arithmetic mean of all measurements was calculated. The results are presented as percent MTT reduction relative to the absorbance of control cells without sample addition. Normally distributed data were analyzed with a one-way analysis of variance (ANOVA) using a Tukey post-hoc test.

2.3 Incubation of peptides in different media simulating the gastrointestinal tract, blood and liver as well as sample preparation for RP-HPLC and UHPLC-ESI-QTOF-MS analysis

The preparation of the media was performed as described before in Elfgen et al. (Elfgen et al., 2017). Preparation of simulated gastric and intestinal fluid (SGF & SIF) was done according to the European Pharmacopoeia 7.0. Human plasma was obtained from BioTrend (Cologne, Germany) with K3 ethylenediaminetetraacetate (EDTA) as anticoagulant. Pooled human liver microsomes were purchased from Sekisui XenoTech (Kansas City, USA; H1000, Lot: 0710494) and diluted in a NADPH regenerating system.

150 µM of the folic acid RD2 conjugates was incubated in SGF, SIF, human blood plasma and human liver microsomes at 37 °C with gentle shaking for different time periods in triplicate. As a reference value for the unmetabolized conjugate at time point zero, the peptides were added to the media and the incubation was stopped immediately. The incubation was stopped by precipitating the proteins with 3% (w/v) trichloroacetic acid (TCA). For the extraction of the folic acid RD2 conjugates and their cleavage products, the samples were centrifuged at 14,000 g and 4 °C for 5 min. The supernatant containing the peptides (100 µM) was collected and stored at -80 °C until analysis. Precipitated medium without peptides served as a control. In addition, the peptides were incubated in distilled water for 0 and 24 h to consider the influence of possible instability at 37 °C. For the quantification of RD2 by LC-MS, internal standard (final concentration: 16.7 ng/mL) was added to the plasma samples.

2.4 Intestinal permeability test

The permeability of FA(t)RD2 and RD2 was investigated in an *in vitro* intestinal epithelial model – the Caco-2 cell monolayer. The assays were performed by Eurofins Panlabs Inc (St Charles, USA). Caco-2 cells are human epithelial colorectal adenocarcinoma cells that differentiate in culture. The cell line expresses, among others, the FRα (Doucette and Stevens, 2001).

The cells were seeded at $1x10^5$ cells/cm^2 in 96-well Multiscreen plates (Millipore) and the permeability assay was performed at days 21 to 25 post-seeding. The test compound was dissolved in water and subsequently added to the donor side at 10 µM in HBSS-HEPES (pH 7.4). The same buffer conditions were used on the receiver side. The assay was performed in the apical to basolateral (A-B) direction. The assay plate was incubated at 37 °C with gentle shaking for 60 min. Additionally, A-B permeability was assessed in the presence of folic acid (100 µM) in order to examine whether folic acid competes with FA(t)RD2 for the binding at folic acid transporters. Samples were taken from the donor side at the assay start time and the end point as well as from the receiver side at the end point.

Samples were analyzed by UHPLC-MS/MS using selected reaction monitoring. The UHPLC system consisted of a binary LC pump with an autosampler. A C-18 column with a solvent gradient was used to separate RD2 and FA(t)RD2 from buffer components and possible impurities. The MS System was an AB Sciex 5500 triple-quadrupole systems with electrospray ionization.

The reference compounds propranolol (highly permeable), labetalol (moderately permeable) and ranitidine (poorly permeable) were included in each assay to ensure the assay validity. Additionally, fluorescein permeability was assessed as cell monolayer integrity marker after each permeability assay. Cell monolayers with a fluorescein permeability of less than $1.5x10^{-6}$ cm/s were considered intact.

The apparent permeability coefficient ($P_{app;}$ expressed as 10^{-6} cm/s) of the test compound and its recovery were calculated as follows:

$$\mathrm{Papp}\left(\frac{\mathrm{cm}}{\mathrm{s}}\right) = \frac{V_R * C_{R,\,end}}{\Delta t} * \frac{1}{A * (C_{D,\,mid} - C_{R,\,mid})}$$

$$\mathrm{Recovery}\ (\%) = \frac{\mathrm{V_D} * \mathrm{C_{D,\,end}} + \mathrm{V_R} * \mathrm{C_{R,\,end}}}{\mathrm{V_D} * \mathrm{C_{D,\,0}}} * 100$$

A: surface area of the cell monolayer (0.11 cm^2); C: concentration of the test compound, expressed as peak area; D: donor; R: receiver; 0, mid, and end: time zero, mid-point and end of the incubation; Δt: incubation time; V: volume of the donor or receiver.

2.5 Intravenous injection and oral administration of the conjugates in mice

FA(t)RD2 or FA(p)RD2 were each administered intravenously (i.v.) in three male wild-type C57BL/6J mice (12 weeks). The applied concentration of the conjugates was adjusted to be equivalent to an RD2 concentration of 3.3 mg/kg, which means that 4.29 mg/kg FA(t)RD2 or 4.19 mg/kg FA(p)RD2 was administered. 100 mg/kg FA(t)RD2 was furthermore administered orally (p.o.) in eight male wild-type C57BL/6J mice (12 weeks) with a concentration equivalent to 77 mg/kg RD2. The conjugates were dissolved in 0.9% sodium chloride solution and injected into the tail vein or given orally via gavage. 60 min (three mice i.v. and four mice p.o.) and 120 min (four mice p.o.) after administration, the mice were anesthetized with isoflurane and blood samples were taken with a heparin-containing syringe by cardiac puncture. Subsequently, the mice were finalized. The blood was centrifuged at 3,000 g and 4 °C for 5 min to obtain cell-free plasma.

In order to quantify RD2 in the extracted plasma samples, internal standard (final concentration: 16.7 ng/mL (i.v. samples) or 66.6 ng/mL (p.o. samples)) was added to the plasma samples. Plasma of untreated mice as well as the used stock solutions with and without internal standard served as control. All samples were precipitated as previously described and the conjugates, their cleavage products and the internal standard were extracted. The plasma samples of the treated mice were extracted three times each. The supernatant was removed and stored at -80 °C until analysis.

2.6 Quantification of the conjugates and RD2 by RP-HPLC

The stability of the conjugates in simulated gastric and intestinal fluids, human plasma and human liver microsomes (see section 2.3) and purity (see Fig. S1 C & D) were determined by reversed-phase high-performance liquid chromatography (RP-HPLC). The HPLC system (Agilent Technologies, Santa Clara, U.S.A., 1200 series) consisted of a manual injector, a quaternary pump, a temperature-controlled column oven and a variable wavelength detector. Chromatography was carried out with a C18 column (Agilent Technologies, Santa Clara, USA; ZORBAX 300SB-C18 5 µm, 4.6 x 250 mm) at 25 °C and 214 nm with a flow rate of 1 mL/min. The injection volume of the samples was 20 µL. The mobile phases for the stability measurements consisted of (A) acetonitrile with 0.15% (v/v) trifluoroacetic acid (TFA) and (B) distilled water with 0.15% TFA. Run conditions were chosen to separate the two isomers of the conjugates from each other, from cleaved RD2 and from the extracted components of the medium (Table 1). The mobile phases for the determination of the purity consisted of (A) acetonitrile with 0.1% (v/v) TFA and (B) distilled water with 0.1% TFA. Run conditions were chosen to separate the two isomers of the conjugates from each other (Table 2). The

chromatograms were recorded and analyzed using the Agilent Software ChemStation (G2175BA, B03.01). The sum of the peak areas of the two unmetabolized conjugate isomers after different incubation times was normalized to the sum of the peak areas of the two conjugate isomers after immediate extraction. The peak area of the emerging RD2 was normalized to the RD2 peak area, which is detected when RD2 is in the same molar concentration as the conjugates at time point zero (100 µM). Normalized peak areas of each triplet were averaged and data presented as mean ± standard deviation.

Table 1: RP-HPLC run conditions for the separation of the two isomers of folic acid RD2 conjugates and RD2. Mobile phase A: acetonitrile with 0.15% TFA. Mobile phase B: water with 0.15% TFA.

	FA(t)RD2			FA(p)RD2		
Step	**Time [min]**	**Mobile phase A [%]**	**Mobile phase B [%]**	**Time [min]**	**Mobile phase A [%]**	**Mobile phase B [%]**
1	0	10	90	0	10	90
2	11	10	90	11	10	90
3	19	14	86	21	15	85
4	33	14	86	40	15	85

Table 2: RP-HPLC run conditions for the determination of the purity of FA(t)RD2 and FA(p)RD2.
Mobile phase A: Acetonitrile with 0.1 % TFA. Mobile phase B: Water with 0.1 % TFA.

Step	**Time [min]**	**Mobile phase A [%]**	**Mobile phase B [%]**
1	0	5	95
2	40	20	80

2.7 Mass determination and quantification of the conjugates and metabolites by UHPLC-ESI-QTOF-MS

A portion of the samples of the FA(t)RD2 incubated *in vitro* (see section 2.1 and 2.3) was additionally analyzed using ultra-high performance liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (UHPLC-ESI-QTOF-MS) to determine the identity of the peptides and to look for potential metabolites. Therefore, the samples were diluted 1:50 in a water/acetonitrile/formic acid mixture (85/15/0.1% (v/v)). In addition, the murine plasma samples of the *in vivo* metabolized conjugates (see section 2.4) were analyzed by this method. Differences in the ionization strength of FA(t)RD2 or FA(p)RD2 and RD2 were determined by comparing the peak areas of equal peptide concentrations. At equal concentrations, RD2 ionizes by factor 2.9 and 6.4 higher than FA(t)RD2 and FA(p)RD2, respectively. Ideally, the ionization efficiency should be investigated in all media because of

possible matrix effects. However, for a qualitative review of metabolization without quantification, the calculated factor for water was sufficient.

The monoisotopic mass of FA(t)RD2, FA(p)RD2, RD2 with residual glycine linker (G-RD2), RD2 and the internal standard was determined as 2079.06 Da, 2021.05 Da, 1651.90 Da, 1597.91 Da and 1583.90 Da, respectively. RD2 was detected exclusively with *m/z* 533.64^{3+}, G-RD2 with *m/z* 551.63^{3+} and the internal standard with *m/z* 528.98^{3+} corresponding to the three times charge state $(M+3H)^{3+}$ of the peptides. FA(t)RD2 and FA(p)RD2 were detected in the three times (*m/z* 693.69^{3+} and *m/z* 674.69^{3+}) and four times (*m/z* 520.52^{4+} and *m/z* 506.26^{4+}) charged species. G-RD2, which was not referenced, was assessed as similar to RD2.

FA(t)RD2, RD2 and G-RD2 were quantified in plasma samples after oral administration. The FA(t)RD2 concentration was externally determined based on a calibration curve. The RD2 concentration was calculated based on the determined concentrations of the internal standard added before extraction.

The UHPLC-ESI-QTOF-MS analysis was performed according to Hupert et al. (2018) except for the running conditions for FA(t)RD2 and FA(p)RD2 which are listed in Table 3. The flow rate was 0.5 mL/min. The detection was carried out with a QTOF detector in ESI-positive ionization mode in a mass range of *m/z* 100-1000.

Table 3: UHPLC running conditions for the detection of the conjugates, impurities and metabolites. Mobile phase A: acetonitrile with 0.025% HFBA and 1% formic acid. Mobile phase B: water with 0.025% HFBA and 1% formic acid. Flow rate: 0.5 mL/min.

Step	Time [min]	Mobile phase A [%]	Mobile phase B [%]
1	0.0	10	90
2	0.5	10	90
3	0.6	16.5	83.5
4	25	16.5	83.5

3. Results

3.1 Both conjugates do not influence cell viability and reduce $A\beta_{1-42}$-induced cytotoxicity

In order to determine whether FA(t)RD2 and FA(p)RD2 have any cytotoxic potential and whether they equally reduce the $A\beta_{1-42}$-induced cytotoxicity, as previously shown for

unconjugated RD2 (van Groen et al., 2017), a MTT cell viability test with PC12 cells was performed. The test was additionally repeated with RD2 to obtain a direct reference value.

The results showed that the cell viability did not significantly change with the addition of 5 µM FA(t)RD2, FA(p)RD2 or RD2 compared to the buffer control and thus the peptides do not have any influence on the cell viability (Fig. 2). In addition, FA(t)RD2, FA(p)RD2 and RD2 reduced the cytotoxicity of aggregated $A\beta_{1-42}$ significantly (FA(t)RD2 and RD2 = $p \leq 0.001$; FA(p)RD2 = $p \leq 0.05$). The cell viability of $A\beta_{1-42}$ alone was 36 ± 3%, whereas the cell viability after co-incubation of $A\beta_{1-42}$ with FA(t)RD2, FA(p)RD2 and RD2 was 55 ± 16%, 48 ± 17% and 64 ± 19%, respectively.

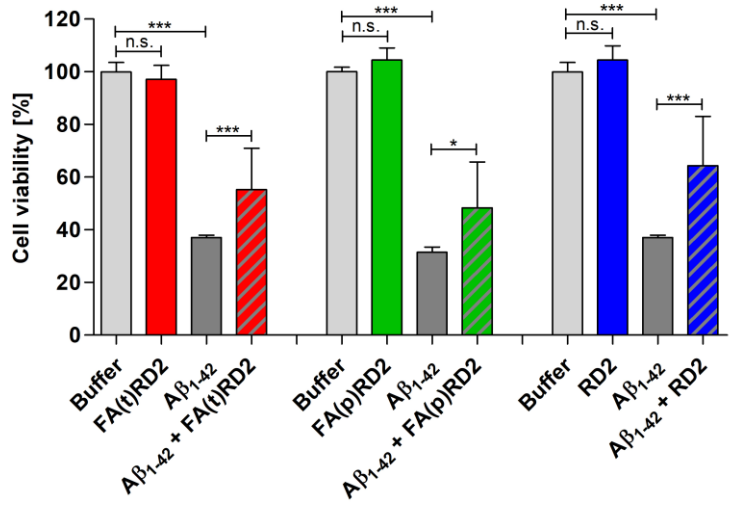

Fig. 2: Cell viability test with FA(t)RD2, FA(p)RD2, RD2 and $A\beta_{1-42}$. The cytotoxic potential of FA(t)RD2, FA(p)RD2 and RD2 as well as their influence on $A\beta_{1-42}$-induced cytotoxicity was investigated using MTT cell viability test with PC12 cells. 5 µM FA(t)RD2, FA(p)RD2 and RD2 or 1 µM aggregated $A\beta_{1-42}$, pre-incubated with and without 5 µM FA(t)RD2, FA(p)RD2 or RD2, was incubated with the cells. The extinction under addition of the peptides was normalized to the extinction without addition of the peptides (buffer). FA(t)RD2, FA(p)RD2 and RD2 were not cytotoxic and reduced the negative impact of aggregated $A\beta_{1-42}$ on cell viability significantly. The data are presented as mean ± standard deviation. Statistics: one factorial ANOVA with Tukey post hoc test; *: $p \leq 0.05$; ***: $p \leq 0.001$; n.s.: not significant.

3.2 FA(t)RD2 is preferentially metabolized in blood plasma while FA(p)RD2 remains unaltered

To verify that the two conjugates, FA(t)RD2 and FA(p)RD2, remain stable in the gastrointestinal tract, but are metabolized to the unconjugated drug, i.e. RD2 without ligand, in the blood or the liver, the peptides were incubated in SGF, SIF, human blood plasma and human liver microsomes. After extraction from the media, both isomers of the conjugates were separated from each other, cleaved RD2, further metabolites and remaining components of the media via RP-HPLC. Hereafter, the percentage of the sum of the two unmetabolized

conjugate isomers is always indicated. However, in Fig. 3 and 4, the isomers are presented separately to show possible differences in metabolism behavior.

To determine the extraction efficiency of the conjugates and RD2 from SGF, SIF, human plasma and human liver microsomes, the peak area of the peptides after extraction from the media was compared to the peak area of the peptides in water. Table 4 shows an overview of the results.

Table 4: Extraction efficiency of both conjugates and RD2 from SGF, SIF, plasma and liver microsomes.

	FA(t)RD2	FA(p)RD2	RD2
	Extraction efficiency [%]		
SGF	94	99	86
SIF	80	85	87
Plasma	67	72	92
Liver microsomes	85	72	83

In SGF, FA(t)RD2 remained almost completely stable over 24 h (82.0 ± 4.2%). Only 5.5 ± 0.8% RD2 was released (Fig. 3 A). In SIF, FA(t)RD2 was metabolized by 16.9 ± 3.0% within 2 h, by 40.3 ± 2.1% within 8 h and by 67.3 ± 1.4% within 24 h, with up to 41.1 ± 2.9% formed RD2 after 24 h (Fig. 3 B). In plasma, the conjugate was metabolized more rapidly to RD2, so that 77.0 ± 1.6% of the conjugate was already degraded after 8 h and 47.7 ± 1.8% RD2 were formed (Fig. 3 C). Also in the liver microsomes, the folic acid was increasingly cleaved from RD2, so that only 32.5 ± 0.8% of the conjugate could be detected after 24 h and 24.3 ± 1.2% RD2 were formed (Fig. 3 D). In SGF, plasma and liver microsomes, no difference in metabolism between the two conjugate isomers could be detected. In SIF, there were small differences in the rate of metabolism, which, however, became relative again after 24 h. In water without addition of enzymes, the conjugate remained almost completely stable for 24 h and < 5% RD2 was formed (Fig. S2 A).

The recovery rate for remaining FA(t)RD2 added with cleaved RD2 has never been 100%. Considering the stability of the unconjugated RD2 in these (Elfgen et al., 2019), it can be assumed that the formed RD2 in SGF and SIF was not further degraded in the time period studied, while for plasma and liver microsomes it must be assumed that a small proportion of the cleaved RD2 was metabolized again during further incubation. In addition, sample analysis by LC-MS showed that not only RD2 but also small amounts of G-RD2 were formed (Table 5).

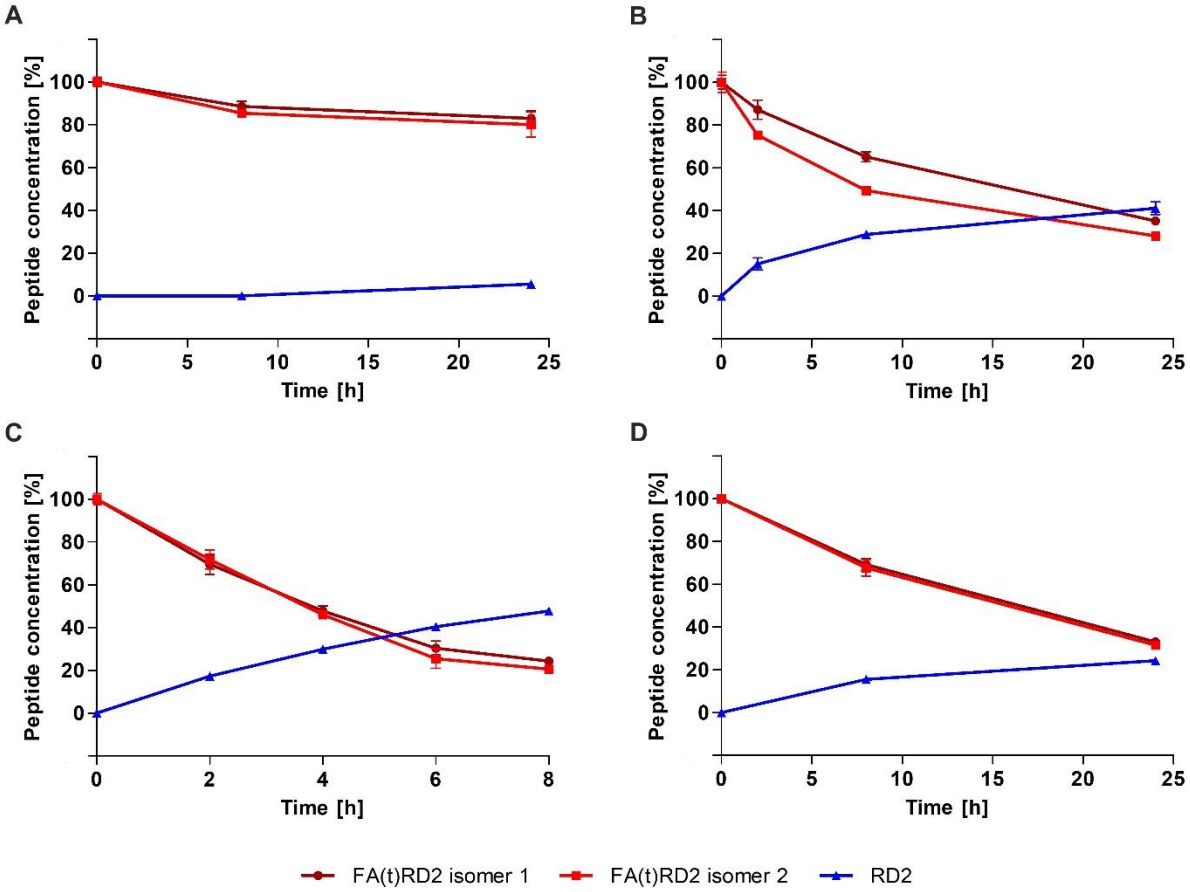

Fig. 3: Metabolization of FA(t)RD2 to RD2 in SGF, SIF, plasma and liver microsomes. FA(t)RD2 was incubated in simulated gastric fluid (SGF) **(A)**, simulated intestinal fluid (SIF) **(B)**, blood plasma **(C)** and liver microsomes **(D)** and the metabolization to RD2 was analyzed by RP-HPLC. The two isomers could be separated and are referred to as isomer 1 and 2. FA(t)RD2 remained almost completely stable in SGF, while it was increasingly metabolized to RD2 in SIF, plasma and liver microsomes. FA(t)RD2 was most rapidly degraded in plasma. The data are presented as mean ± standard deviation (n = 3).

Table 5: Approximate molar ratio of FA(t)RD2 to RD2 and G-RD2 in SGF, SIF, plasma and liver microsomes. The 2.9-fold increased ionization of RD2 and G-RD2 towards FA(t)RD2 was included in the calculations.

	Molar ratio	
	FA(t)RD2 / RD2	**FA(t)RD2 / G-RD2**
SGF, 24 h	25.3	1589.6
SIF, 8 h	2.8	224.9
Plasma, 8 h	0.5	34.0
Liver microsomes, 24 h	1.9	13.3

The second conjugate, FA(p)RD2, remained almost completely stable in SGF, SIF and liver microsomes for 24 h and in plasma even over 3 days (88.8, 88.3, 82.8 and 82.7%, respectively) and was not metabolized to RD2 (Fig. 4). In water without addition of enzymes, the conjugate remained completely stable for 24 h (Fig. S2 B). There was no difference in the metabolization behavior of the two conjugate isomers in any of the media.

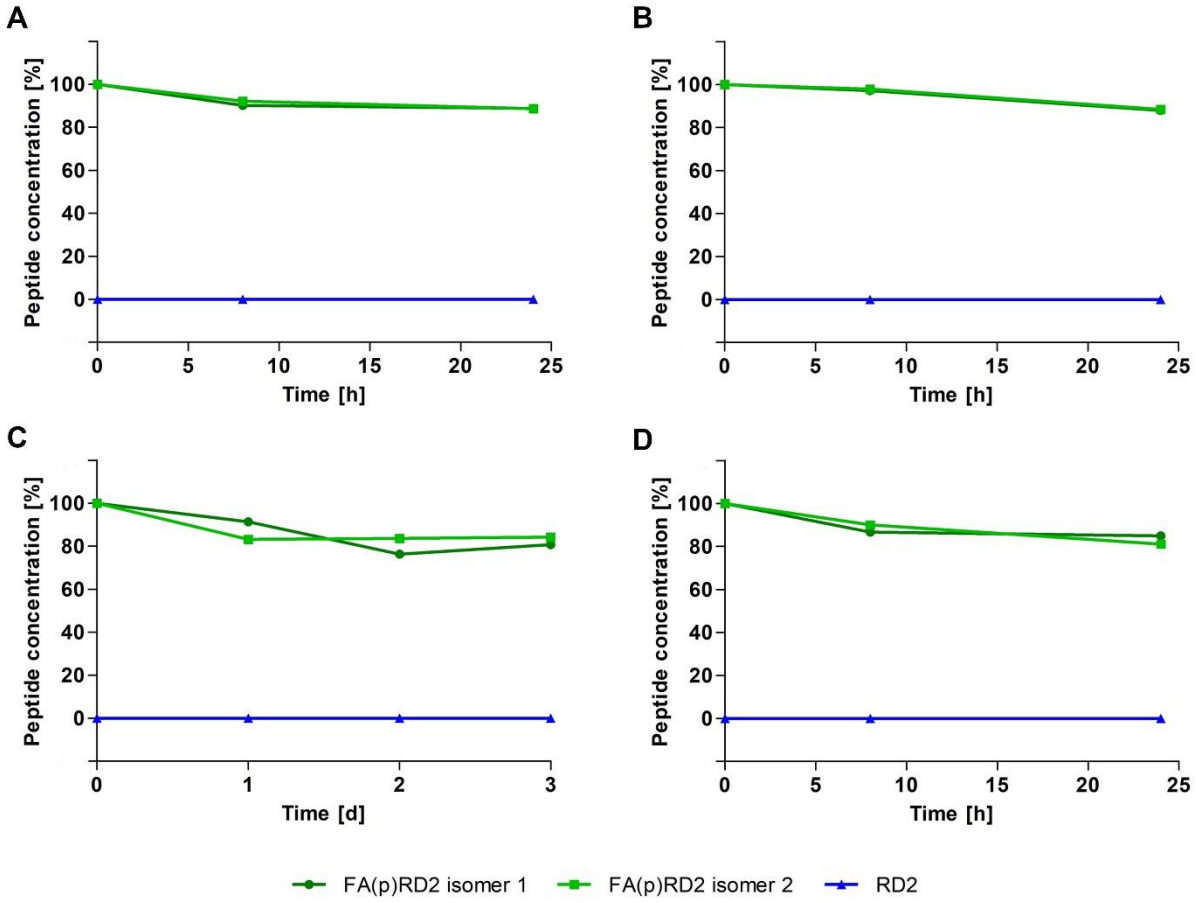

Fig. 4: Metabolization of FA(p)RD2 to RD2 in SGF, SIF, plasma and liver microsomes. FA(p)RD2 was incubated in simulated gastric fluid (SGF) **(A)**, simulated intestinal fluid (SIF) **(B)**, blood plasma **(C)** and liver microsomes **(D)** and the metabolization to RD2 was analyzed by RP-HPLC. The two isomers could be separated and are referred to as isomer 1 and 2. FA(p)RD2 remained almost completely stable in all media over the incubated time. RD2 has not been released. The data represent simple determinations.

3.3 In contrast to FA(p)RD2, FA(t)RD2 is metabolized to RD2 after i.v. injection in mice

The metabolization of both conjugates to the unconjugated drug RD2 was additionally investigated *in vivo* in mice. For this purpose, both conjugates were injected i.v. (4.3 mg/kg FA(t)RD2 or 4.2 mg/kg FA(p)RD2, equivalent to 3.3 mg/kg molar concentration of RD2) and the conjugates as well as RD2 and G-RD2 were analyzed by LC-MS in blood plasma samples

taken 60 min after administration. The RD2 concentration was calculated based on the determined concentrations of the internal standard added before extraction.

After FA(p)RD2 injection, solely folic acid conjugated RD2 could be detected in plasma, meaning that FA(p)RD2 was not metabolized to RD2 within 60 min.

For FA(t)RD2, metabolization to RD2 has been detected, but no formation of G-RD2. However, FA(t)RD2 was not completely metabolized within 60 min. The ratio of FA(t)RD2 to RD2 was approximately 9:1 (ionization factors of the peptides included). The amount of RD2 contained in the plasma samples after 60 min was determined as 17.5 ± 3.9 nM (Table 6).

Table 6: Detection of FA(t)RD2 and RD2 in mouse plasma 60 min after i.v. injection of 4.29 mg/kg FA(t)RD2. The samples were extracted three times each, analyzed by LC-MS and the peak areas were calculated. The ratio between FA(t)RD2 and RD2 is the peak area of FA(t)RD2 divided by the peak area of RD2. Quantification of the cleaved RD2 was done using the internal standard.

	Mouse 1	**Mouse 2**	**Mouse 3**
	Calculated quantity ratios		
FA(t)RD2 / RD2	10.0 ± 1.3	8.7 ± 0.6	7.5 ± 1.3
	Calculated concentration [mean ± SD in nM]		
RD2	19.3 ± 3.7	15.9 ± 3.7	17.3 ± 4.3

SD = standard deviation

3.4 The intestinal absorption of FA(t)RD2 is increased compared to unconjugated RD2

The intestinal absorption of FA(t)RD2 and RD2 was compared in an FRα-expressing cell model of the intestinal barrier (Caco-2). Unconjugated RD2 had a low permeability of only 0.07 ± 0.03 x 10^{-6} cm/s. In contrast, folic acid conjugated RD2 (FA(t)RD2) showed a significantly increased (7-fold) permeability towards unconjugated RD2 (0.47 ± 0.03 x 10^{-6} cm/s) (Fig. 5). In the presence of folic acid, the permeability is significantly decreased (0.01 ± 0.01 x 10^{-6} cm/s) indicating that folic acid competes with FA(t)RD2 for the binding at folic acid transporters mediating the transport across the intestinal barrier. The recovery of both compounds was between 53 and 61%.

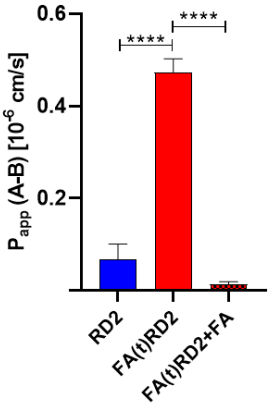

Fig. 5: Permeability of RD2 and FA(t)RD2 in FRα expressing Caco-2 cell monolayers. Permeability of RD2 and FA(t)RD2 alone and in the presence of folic acid (FA). Statistics: one way ANOVA; ****: $p \leq 0.0001$.

3.5 The oral absorption of RD2 can be increased by folic acid conjugation

The oral absorption of FA(t)RD2 was investigated by administering 100 mg/kg FA(t)RD2 (equivalent to 77 mg/kg RD2) peroral in mice for one and two hours. The conjugate, RD2 and G-RD2 were subsequently analyzed by LC-MS in blood plasma samples.

60 min after p.o. administration of FA(t)RD2, 53 ± 5 or 61 ± 5 nM RD2 and 0.92 ± 0.24 or 1.21 ± 0.16 µM FA(t)RD2 was detected in two out of four blood samples. However, in 50% of the samples the concentration of FA(t)RD2 or RD2 was below the detection limit (Table 7).

There was no difference in oral absorption noticeable between the 60 min and the 120 min time point. 120 min after FA(t)RD2 administration, the RD2 concentration was also not detectable in two samples, was only 21 ± 2 nM in the third sample and was even 189 ± 18 nM in the fourth sample. FA(t)RD2 was only detectable in one out of four samples with a concentration of 1.84 ± 0.33 µM.

The total molecular RD2 concentration calculated by the sum of unconjugated RD2 and FA(t)RD2 was 0.98 ± 0.24 and 1.27 ± 0.17 µM after 60 min and 21 ± 2 nM and 2.02 ± 0.35 µM after 120 min.

While in half of the samples no RD2 or FA(t)RD2 could be detected, in the other half of samples the overall RD2 concentration in the blood was enhanced upon 100 mg/kg p.o. FA(t)RD2 administration (equivalent to 77 mg/kg RD2) compared to a previously conducted p.o. study with 200 mg/kg unconjugated RD2 (unpublished data). In this study, the RD2 concentration in the three plasma samples 60 min after administration was 432 ± 17, 237 ± 11 and 106 ± 10 nM. Converted to 77 mg/kg RD2 this corresponds to 166, 91 and 41 nM assuming a linear process. Compared to our study with p.o. administered FA(t)RD2 (60 min) this means that FA(t)RD2 enhances the oral absorption by factor 6 to 31.

One major downside of the oral FA(t)RD2 study was that half of the mice got lethargic a few minutes after administration for several minutes up to half an hour. No correlation between the lethargic mice and the samples without detectable FA(t)RD2 and RD2 could be ascertained.

Table 7: Detection of FA(t)RD2 and RD2 in mouse plasma 60 and 120 min after p.o. administration of 100 mg/kg FA(t)RD2. The samples were extracted three times each, analyzed by LC-MS and the peak areas were calculated. Cleaved RD2 was calculated using the internal standard. The FA(t)RD2 concentration was determined by a FA(t)RD2 calibration curve.

Time	**60 min**				**120 min**			
Mouse	1	2	3	4	1	2	3	4
	Calculated concentrations [mean ± SD in nM]							
FA(t)RD2	-	922 ± 239	-	1208 ± 162	-	-	-	1835 ± 333
RD2	-	53 ± 5	-	61 ± 5	-	21 ± 2	-	189 ± 18
Sum	-	975 ± 243	-	1269 ± 166	-	21 ± 2	-	2024 ± 351

- = not detectable; SD = standard deviation

4. Discussion

In previous studies, it has already been shown that the D-peptide RD2 is suitable for oral administration as it exhibits a remarkably high enzymatic resistance in the gastrointestinal tract, the blood and the liver (Elfgen et al., 2019), and a high oral bioavailability (Leithold et al., 2016) so it is effective after oral administration (Kutzsche et al., 2017; Schemmert et al., 2019a; Schemmert et al., 2019b). However, we were interested in increasing RD2's intestinal absorption to achieve an enhanced efficacy upon similar dosage or to allow for a lower dosage with equal efficacy.

In recent years, several successful studies on the endocytosis of folic acid-conjugated drugs into different tissue and tumor cells have been performed (Hilgenbrink and Low, 2005; Leamon, 2008), including studies on increasing the oral bioavailability (Anderson et al., 2001; Roger et al., 2012). The latter showed that uptake of orally administered liposomes or nanoparticles can be increased by conjugation with folic acid presumably mediated by endocytosis via the folate receptor (Kamen and Capdevila, 1986; Leamon and Low, 1991; Zhao et al., 2011). Based on this idea, RD2 has been conjugated with folic acid in two different ways: 1) direct coupling of folic acid's carboxyl groups to RD2's proline (position 1) forming a relatively stable amide bond (FA(p)RD2) and 2) indirect coupling of folic acid's carboxyl groups to RD2's threonine (position 2) by a glycine linker forming a weak ester bond between glycine

and threonine (FA(t)RD2). The conjugates were designed to be ideally stable in the gastrointestinal tract and to release folic acid from RD2 only in the intestinal epithelial cells or in the blood and liver. This is to ensure that RD2 retains its unaltered efficacy and blood brain barrier permeability.

First of all, it could be shown that both conjugates are not cytotoxic per se in concentrations of 5 µM and that the cytotoxic potential of $A\beta_{1-42}$ might still be reduced by FA(t)RD2 and FA(p)RD2, as it has previously already been shown for unconjugated RD2, indicating that even if the conjugates reached the brain unmetabolized, RD2's mode of action would be retained.

We investigated FA(t)RD2's and FA(p)RD2's metabolization to the unconjugated drug in simulated gastric and intestinal fluids (SGF & SIF), human blood plasma and human liver microsomes *in vitro* as well as in blood samples taken after i.v. injection in mice. Both conjugates remained almost completely stable in SGF for 24 h. In SIF, FA(p)RD2 also showed very high stability and no RD2 cleavage. FA(t)RD2 was degraded by 17% after 2 h with increasing formation of RD2. Since the residence time of particles in the small intestine is 1 to 3 h (Dressman et al., 1998), it can be assumed that FA(t)RD2 would still pass through the small intestine almost completely unmetabolized towards the colon. In the colon itself, the two conjugates are mainly exposed to bacterial and enzymatic degradation by exoenzymes, whereby the residence time in the colon can be from hours to days (Dressman et al., 1998), so that there is sufficient time for the uptake via the FRα expressed in this intestinal region (Holm et al., 1994; Parker et al., 2005). Studies on the stability of FA(t)RD2 in human blood plasma and human liver microsomes showed that the conjugate was mainly metabolized to unaltered RD2 without glycine linker, in plasma by 80% within 8 h, in liver microsomes slightly slower. In plasma samples taken from mice 60 min after i.v. injection of FA(t)RD2, RD2 could be detected in a ratio of 9:1 FA(t)RD2 to RD2. In contrast, folic acid has not been cleaved from FA(p)RD2 *in vitro* in blood plasma and liver microsomes as well as after i.v. injection.

As FA(t)RD2 seemed to be appropriate as prodrug, it has been further tested if the conjugation yields an advantage for RD2's intestinal absorption mediated by folic acid transporters in the intestinal wall. In the Caco-2 cell model emulating the intestinal barrier, FA(t)RD2 showed a 7-fold enhanced permeability in contrast to unconjugated RD2. Additional experiments in the presence of folic acid indicated that FA(t)RD2's absorption is indeed mediated by folic acid transporters, most presumably by the FRα, which is the only folic acid transporter in the intestine that allows the transport of large molecules across membranes by endocytosis (Parker et al., 2005; Zhao et al., 2011; Zhao et al., 2009).

Our study with orally administered FA(t)RD2 in mice showed that the conjugation with folic acid promotes the intestinal RD2 absorption. Compared to a study with orally administered unconjugated RD2 (unpublished data) the administration of the folic acid conjugate led to an 7.5- to 40-fold increased RD2 concentration in the blood after 60 min. However, the intestinal absorption of FA(t)RD2 varied strongly between samples of the same time point and no trend of absorption quantity between 60 min and 120 min could be detected. Thus, the intestinal absorption seems to strongly depend on physical conditions, e.g. activity and food intake. Although a repetition of the study with more time points, different FA(t)RD2 concentrations and/or fasted mice could have brought clarification, we decided to terminate the study as half of the treated mice got lethargic shortly after oral administration of 100 mg/kg FA(t)RD2 for approx. 30 min. Although it is known from the relevant literature that the C57BL/6 mouse strain have a relatively low LD_{50} value for folic acid (i.p. LD_{50}: 85 to 100 mg/kg) which reveals particular susceptibility of this specific strain to folic acid toxicity (Parchure et al., 1985) we decided to still use C57BL/6 mice in order to ensure the comparability between the previously performed RD2 study and the current FA(t)RD2 study. The observed lethargic behavior of the mice after FA(t)RD2 treatment is in line with symptoms (ataxia and muscular weakness) described in the study by Parchure et al. (1985) where unconjugated folic acid was administered intraperitoneally. Several further facts speak against a toxicity of the conjugate and for a strain-specific reaction with high compound concentrations: both conjugates and RD2 have been shown to not affect PC12 cell viability, RD2 alone was well tolerated in mice in concentrations up to 200 mg/kg/day for up to three month (Schemmert et al., 2019), the tolerable upper intake limit of folic acid in human has been assessed as 1 mg/day or even higher (Institute of Medicine, 1998) and folic acid has been shown to support PC12 cell viability in concentrations up to 1 g/L (Kim et al., 2018; Kim and Yang). Moreover, due to the enhanced absorption of the prodrug the dosage could be reduced with unchanged efficacy which should counteract toxicity possibly resulting from high folic acid concentrations.

Conclusion

The experiments show that FA(t)RD2 could be suitable as prodrug since it has an increased intestinal absorption in comparison to unconjugated RD2, it shows a high stability in the gastrointestinal tract and the ligand is cleavable from the drug after systemic uptake to ensure the known efficacy of unaltered RD2. However, higher oral doses (100 mg/kg) of the prodrug FA(t)RD2 may not be suitable as undesired effects may outweigh the desired efficacy. In contrast, the bond between folic acid and RD2 of the FA(p)RD2 conjugate is too stable to be systematically cleaved, which makes FA(p)RD2 inappropriate as prodrug.

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Declarations of interest

None

Submission Declaration and Verification

The authors guarantee that the manuscript describes original work, is not under consideration for publication elsewhere and has not been published 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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