Research Article

Astrocyte-Targeted Transporter-Utilizing Derivatives of Ferulic Acid Can Have Multifunctional Effects Ameliorating Inflammation and Oxidative Stress in the Brain

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Ferulic acid (FA) is a natural phenolic antioxidant, which can exert also several other beneficial effects to combat neuroinflammation and neurodegenerative diseases, such as Alzheimer’s disease. One of these properties is the inhibition of several enzymes and factors, such as β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1), cyclooxygenases (COXs), lipoxygenases (LOXs), mammalian (or mechanistic) target for rapamycin (mTOR), and transcription factor NF-κB. We have previously synthesized three L-type amino acid transporter 1- (LAT1-) utilizing FA-derivatives with the aim to develop brain-targeted prodrugs of FA. In the present study, the cellular uptake and bioavailability of these FA-derivatives were evaluated in mouse primary astrocytic cell cultures together with their inhibitory effects towards BACE1, COX/LOX, mTOR, NF-κB, acetylcholinesterase (AChE), and oxidative stress. According to the results, all three FA-derivatives were taken up 200–600 times more effectively at 10 μM concentration into the astrocytes than FA, with one derivative having a high intracellular bioavailability (Kpu,u), particularly at low concentrations. Moreover, all of the derivatives were able to inhibit BACE1, COX/LOX, mTOR, NF-κB, acetylcholinesterase (AChE), and oxidative stress measured as decreased cellular lipid peroxidation. Furthermore, one of the derivatives modified the total mTOR amount. Therefore, these derivatives have the potential to act as multifunctional compounds preventing β-amyloid accumulation as well as combating inflammation and reducing oxidative stress in the brain. Thus, this study shows that converting a parent drug into a transporter-utilizing derivative not only may increase its brain and cellular uptake, and bioavailability but can also broaden the spectrum of pharmacological effects elicited by the derivative.

1. Introduction

Most brain disorders and diseases lack effective drug therapies [1–3]. Current treatments of cerebral viral and bacterial infections as well as drugs against neurodegenerative and autoimmune diseases, such as Parkinson’s disease (PD), Alzheimer’s disease (AD), Huntington’s disease (HD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), epilepsy, stroke, and traumatic brain injury, are largely unsatisfactory, often providing only symptomatic relief. One reason for the poor therapeutic responses in many central nervous system (CNS) diseases and disorders is due to ineffective drug delivery, i.e., the inability of a drug molecule to reach its target site at effective concentrations within the brain [4, 5]. The blood-brain barrier (BBB) prevents many drugs and other xenobiotics from gaining access to the brain, and therefore, several novel drug delivery technologies have been devised to overcome this obstacle. However, despite major industrial and academic efforts to develop novel drug delivery systems, this puzzle has remained largely unresolved. Furthermore, it was proposed over a decade ago that the cellular membranes of
parenchyma cells could serve as a secondary barrier to drug permeability within the brain [6, 7]. However, this aspect has been neglected, even though a significant number of the novel CNS drugs have intracellular targets [8].

Inadequate understanding of the disease’s etiology in the brain and poor translation from the animal models to the human situation have also contributed to the decelerated CNS drug development success rate [4]. Recently, oxidative stress and neuroinflammation have been proposed as potential mechanisms involved in the pathogenesis of several brain disorders and as possible targets in neuroprotective treatments [9, 10]. Since oxidative stress (i.e., overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS)) as well as inflammation (i.e., overproduction of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 as well as the overexpression of iNOS) can directly cause neuronal cell death or trigger a cascade of events that leads to further neurodegeneration (protein misfolding, mitochondrial dysfunction, or glial cell activation), the prevention of increased levels of ROS, RNS, and proinflammatory cytokines can not only treat the symptoms but also affect the progression of the neurodegenerative diseases. Natural polyphenols, which are compounds mainly found in plants, are known to be strong antioxidants and therefore can help prevent intracellular accumulation of ROS. Numerous studies have shown that they can exert multiple neuroprotective effects in neurological disorders and ischemia [11–13]. However, most of these polyphenols, like almost all small-molecular weight drugs, are unable to cross the BBB and reach their intracellular target sites within the brain [14].

One natural phytochemical, ferulic acid (FA) (trans-4-hydroxy-3-methoxycinnamic acid; Figure 1), found in fruits and vegetables, is a phenolic antioxidant [15]. In addition to being a free radical scavenger against the ROS produced during inflammation within the brain, it has also been reported to ameliorate memory deficits in the amyloid-β-(Aβ1-42)-induced Alzheimer’s disease (AD) mouse model by inhibiting β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1) activity [16]. This was found to prevent further Aβ1-42 accumulation and to help to lessen the subsequent activation of astrocytes and microglial cells [17, 18]. We have previously reported that L-type amino acid transporter-1 (LAT1-) utilizing prodrugs of FA can display enhanced delivery across the BBB and thus can be accumulated with the brain [19]. However, a previous study also revealed that the prodrugs that were conjugated to FA via an amide bond (derivatives 1 and 2 in Figure 1) were not capable of releasing FA at a sufficient rate within the mouse brain in vivo. FA-derivative 1 did not release any FA, while FA-derivative 2 released less than 5% of FA in the brain.

The corresponding ester derivative of FA (derivative 3 in Figure 1) had a more favorable bioconversion rate in vitro; in mouse brain S9 subcellular fraction, it released approximately 30% of FA during a 5 h incubation, while derivatives 1 and 3 remained intact. However, due to the very high carboxylesterase activity in the plasma and in the hepatocytes, the ester prodrug was not able to deliver FA into the mouse brain; instead, it was prematurely bioconverted to FA by the first pass metabolism. In contrast, in human plasma and liver S9 subcellular fraction, prodrug 3 was 10 times more stable. Due to their higher stability, previously called prodrugs 1-3 will be considered here as FA-derivatives. Thus, the aim of the present study was to evaluate the detailed transport mechanisms of the LAT1-utilizing derivatives of FA into their target brain cells, namely, mouse primary astrocytes [20], and investigate their effects on inflammation and oxidative stress. This is highly important information in determining whether these compounds could be used as therapeutic agents in their intact form and not as prodrugs.

2. Experiments

2.1. Materials. All reagents and solvents used in analytical studies were commercial and high purity of analytical grade or ultragradient HPLC-grade purchased from Sigma (St. Louis, MO, USA), J.T. Baker (Deventer, The Netherlands), Merck (Darmstadt, Germany), Riedel-de Haën (Seelze, Germany), or Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Water was purified using a Milli-Q Gradient system (Millipore, Milford, MA, USA).

2.2. Cell Cultures. Primary astrocytes from the cortex and hippocampi were isolated from 2-day-old wild-type and APP/PS1 transgenic mice as previously described [21, 22]. Mice carrying human APP (K595N and M596L) and PSEN1dE9 mutations maintained in C57BL/6J background.

Figure 1: Ferulic acid (FA) and its L-type amino acid transporter 1- (LAT1-) utilizing derivatives 1-3.
were used as a mouse model of AD (Jackson Laboratories, Bar Harbor, ME, USA). The animals were housed and treated as described above, and cortices and hippocampi were isolated by suspending the brain tissue into DMEM medium containing 10% heat-inactivated fetal bovine serum and penicillin streptomycin (100 U/mL). The suspension was triturated ten times and thereafter centrifuged at 1500 rpm for 5 min at room temperature. Trypsin-EDTA of 0.25% was added, and the suspension was incubated for 30 min at 37°C. Fresh culture medium was added, and the suspension was centrifuged at 1500 rpm for 5 min. The astrocytes were cultured in Dulbecco’s modified Eagle medium/F-12 Nutrient Mixture (DMEM/F2) supplemented with L-glutamine (2 mM), heat-inactivated fetal bovine serum (10%), penicillin (50 U/mL), and streptomycin (50 μg/mL). The cells were plated on a poly-D-lysine coated flasks in culture medium, and to remove the microglia, the cultures were shaken at 200 rpm for 2 h before the experiments described below were performed. It has been reported earlier that these cultures contain approximately 80% astrocytes (20% microglia) [23].

2.3. Ability of Compounds to Bind to LAT1. For the transporter binding studies, the astrocytes were seeded on 24-well plates with a density of 10 × 10⁴ cells/well three days before the experiments. After removal of the culture medium, the cells were carefully washed with prewarmed HBSS (Hank’s balance salt solution) containing choline chloride (125 mM), KCl (4.8 mM), MgSO₄ (1.2 mM), KH₂PO₄ (1.2 mM), CaCl₂ (1.3 mM), glucose (5.6 mM), and HEPES (25 mM) adjusted to pH 7.4 with 1 M NaOH. The cells were preincubated with 500 μL of prewarmed HBSS at 37°C for 10 min before the experiments. To study the ability of studied compounds to inhibit the uptake of a known LAT1 substrate, the cells were incubated at 37°C for 5 min with an uptake buffer (HBSS, 250 μL) containing 0.76 μM (0.25 mM) or 9.85 MBq/mL of [14C]-L-leucine (PerkinElmer, Inc., Bar Harbor, ME, USA). Subsequently, the cells were washed three times with ice-cold HBSS and lysed with 250 μL of NaOH (0.1 M) for 60 min. The lysates were diluted with acetonitrile (ACN) including the selected internal standard (diclofenac to compounds 1-3 and chloroxazone to FA) with a ratio of 1:3 and centrifuged at 10 000 × g for 10 min. The samples were analyzed by the liquid chromatography mass spectrometry (LC-MS) methods described earlier for derivatives 1-3 and FA with an Agilent 1200 Series Rapid Resolution LC System together with an Agilent 6410 Triple Quadrupole Mass Spectrometer equipped with an electrospray ionization source using a Poroshell 120 EC-C-18 column (50 mm x 2.1 mm, 2.7 μm; Agilent Technologies, Santa Clara, CA) [19]. The concentrations of each compound in cell lysates were calculated from the standard curve that was prepared by spiking known amounts of compounds to ACN including the selected internal standard and then normalized with protein concentration. The protein concentrations on each plate were determined as a mean of three samples by Bio-Rad Protein Assay, based on the Bradford dye-binding method, using bovine serum albumin (BSA) as a standard protein and measuring the absorbance (595 nm) by a multiplate reader (EnVision, PerkinElmer, Inc., Waltham, MA, USA).

2.5. Intracellular Unbound Concentrations of Compounds. The unbound fraction of FA and its derivatives 1-2 was determined at 10 μM in cell homogenate by using a Rapid Equilibrium Dialysis (RED) device (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Briefly, the cell homogenate was prepared from astrocyte cell suspension (10 × 10⁶ cells/mL) in HBSS with a SoniPerp 150 Plus disintegrator (MSE Ltd., London, UK) for 2 s × 3. 100 μL of cell homogenate spiked with the studied compound was added to the reaction chamber, and 350 μL of HBSS buffer was added to the buffer chamber of the RED plate. The dialysis plate was incubated at 37°C for 4 h with shaking. 50 μL of samples was taken from the reaction, and buffer chambers and equal size of buffer or blank homogenate were added, respectively, to yield identical matrices. The proteins were precipitated by adding 100 μL of ice-cold ACN (including the selected internal standard; see above), and the supernatants were collected for LC-MS analysis (see above) after centrifugation at 12 000 × g for 10 min. The unbound drug fraction (f unbound) was calculated as described by Mateus et al. [25]:

\[ f_{\text{unbound}} = \frac{C_{\text{buffer}}}{C_{\text{homogenate}}}, \]  

(1)

where \( C_{\text{buffer}} \) is compound concentration in the buffer chamber and \( C_{\text{homogenate}} \) is the compound concentration in the homogenate chamber and corrected with homogenate dilution factor (D) [26]:

\[ f_{\text{unbound}} = \frac{1}{D \times \left(1/f_{\text{unbound}} - 1\right) + 1}, \]  

(2)

where D was estimated to be 45 for 10 × 10⁶ cells/mL cell suspension according to their weight of the cells.
The drug concentration ratio in astrocytes \((K_p)\) was determined at 0.01-1.0 \(\mu\)M concentrations by comparing the cellular uptake from the cell lysates (0.1 M NaOH) to the concentration detected from the surrounding buffer (HBSS) collected before cell lysis as described by Mateus et al. [25]:

\[
K_p = \frac{A_{cell}}{V_{cell}} \frac{C_{medium}}{C_{medium}},
\]

where \(A_{cell}\) is the amount of compound in the cell lysate (nmol), \(V_{cell}\) is the astrocyte cell volume \((0.05810^{-15} \text{L/cell})\) [27] for \(10 \times 10^4\) cells/well, and \(C_{medium}\) is the compound concentration in the surrounding buffer. The unbound drug concentration ratio \((K_{p,uu})\) was then calculated by multiplying the drug concentration ratio \((K_p)\) by unbound drug fraction \((f_{u,cell})\) as described by Mateus et al. [25]:

\[
K_{p,uu} = K_p \times f_{u,cell}.
\]

2.6. Ability of Compounds to Inhibit Astrocyte Cell Growth. Primary astrocytes were seeded at the density of \(10 \times 10^4\) cells/well onto collagen-coated 96-well plates. The cells were used for the proliferation experiments one day after seeding. Concentrations of 5-400 \(\mu\)M FA or its LAT1-utilizing derivatives 1-3 were added into the growth medium and incubated for 3 days. Each day, the cell viability was determined by resazurin cell proliferation kit (Sigma, St. Louis, MO, USA), which is directly proportional to aerobic respiration and cellular metabolism of cells. The samples were measured fluorometrically by monitoring the increase in fluorescence at \(\lambda_{ex} 560\text{ nm} and \lambda_{em} 590\text{ nm}\) (EnVision, PerkinElmer, Inc., Waltham, MA, USA). The cell viability was also followed by visualizing the wells with microscopy. The ability of compounds to inhibit the viability of the cells was expressed as percentages (%) compared to the untreated controls.

2.7. Ability of Compounds to Inhibit Oxidative Stress and Lipid Peroxidation (Malondialdehyde Formation). Primary astrocytes were seeded at the density of \(2 \times 10^5\) cells/well onto collagen-coated 6-well plates. The cells were used for the experiments 2 days after seeding, and first, they were treated with 0.1 \(\mu\)g/mL lipopolysaccharide (LPS) in prewarmed HBSS buffer (250 \(\mu\)L) at 37°C for 24 h. The control cells were treated with prewarmed HBSS buffer only. The ability of compound to inhibit the oxidative stress was evaluated by adding FA (50 \(\mu\)M) or its derivatives 1-3 in prewarmed HBSS buffer (250 \(\mu\)L) together with LPS and incubating the cells at 37°C for 24 h. Tocopherol (vitamin E) was used as a positive control. Subsequently, the cells were washed with PBS (phosphate-buffered saline), detached from the plate by trypsinization and centrifuged at 1000 \(\times g\) for 5 min. The supernatant was removed, and the cell pellet was then resuspended with 0.1 M MES (2-(N-morpholino)ethanesulfonic acid) buffer (pH 6.0), sonicated for 10 min, and centrifuged at 10 000 \(\times g\) for 15 min at 4°C. The supernatant (100 \(\mu\)L) was collected, and 100 \(\mu\)L glacial acetic acid (50%), 4 \(\mu\)L of butylated hydroxytoluene (0.01%), and finally 100 \(\mu\)L of thiobarbituric acid (4 mM) were added and the mixture was incubated in a thermoshaker at 95°C for 1 h. The mixtures were then centrifuged at 10 000 \(\times g\) for 10 min, and 50 \(\mu\)L of the supernatant was analyzed on a 96-well plate, each experiment as three technical replicates from three biological replicates. The fluorescence was read by the EnVision plate reader (EnVision, PerkinElmer, Inc., Waltham, MA, USA) at \(\lambda_{ex} 532\text{ nm} and \lambda_{em} 553\text{ nm}\). The amount of produced malondialdehyde (MDA) in each experiment was quantified from the standard curve; various amounts (1-800 \(\mu\)M) of MDA and thiobarbituric acid in water were incubated in a thermoshaker at 95°C for 1 h and analyzed simultaneously as duplicates with each studied batch. The protein concentrations on each plate were determined as a mean of three samples by Bio-Rad Protein Assay, based on the Bradford dye-binding method, using BSA as a standard protein and measuring the absorbance (595 nm) by a multiplate reader (EnVision, PerkinElmer, Inc., Waltham, MA, USA). The results were analyzed as \(\mu\)mol of formed MDA per mg protein.

2.8. Ability of Compounds to Inhibit BACE1. A fluorometric assay was used to screen the inhibitory effect of the studied compounds on purified human BACE1 using a fluorescence resonance energy transfer (FRET) peptide technique (SensoLyte® 520 BACE1 Assay kit, AnaSpec, Inc., Fremont, CA, USA) according to the manufacturer’s protocol. Briefly, 1 \(\mu\)M and 10 \(\mu\)M of studied compounds were incubated with the FRET substrate (QXL® 520/HiLyte™ Fluor 488) and active BACE1 enzyme at room temperature for 30 minutes. The sequence of the FRET peptide has been derived from the \(\beta\)-secretase cleavage site of \(\beta\)-amyloid precursor protein (APP) with Swedish mutation, which enhances \(\beta\)-secretase to process APP resulting in an early onset of AD. Thus, active \(\beta\)-secretase cleaved FRET substrate into two separate fragments resulting in the release of HiLyte™ Fluor 488 fluorescence that was measured by the EnVision plate reader (EnVision, PerkinElmer, Inc., Waltham, MA, USA) at \(\lambda_{ex} 490\text{ nm} and \lambda_{em} 520\text{ nm}\). Changes in the amount of this fluorophore caused by the inhibition of \(\beta\)-secretase by the studied compounds (1-10 \(\mu\)M) were compared with the control sample and with a specific inhibitor (0.25 \(\mu\)M provided with the kit) (KTEEISEVN-Sta-VAEF-NH2) [28]. The results were reported as percentages (%), with control sample representing 100% \(\beta\)-secretase activity.

2.9. Ability of Compounds to Inhibit Peroxidase Activity of Cyclooxygenases. A coupled oxidation-reduction fluorometric assay was used to assess the inhibitory effect of compounds (100 \(\mu\)M) together with positive controls on the peroxidase activity of cyclooxygenases (COX) (ab204699 Cyclooxygenase Activity Assay Kit, Abcam, Cambridge, UK). The method has been done and validated according to the manufacturer’s protocol. Briefly, studied compounds (100 \(\mu\)M) and the reaction mixture were incubated with the cell lysates at room temperature and the reaction was initiated by adding arachidonic acid/NaOH solution. The fluorescence was read in a kinetic mode after every 15 seconds for 30 minutes by the EnVision plate reader.
2.10. Ability of Compound to Inhibit the Activity of Acetylcholinesterase and Butyrylcholinesterase. Inhibitory activities of FA and its derivatives 1-3 towards acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were determined with an endpoint enzymatic assay in mouse brain S9 fraction by Ellman’s method by using acetylthiocholine (1 mM) for measuring AChE activity and butyrylthiocholine (1 mM) for measuring BChE activity. Briefly, mouse brain S9 fraction was prepared by homogenizing freshly collected mouse brain with 50 mM Tris-buffered saline (TBS) (pH 7.4) (1:4, w/v), centrifuging the homogenate at 9 000 × g for 20 min at 4°C and collecting the supernatant. The supernatant was then diluted at 1:10 with phosphate-buffered saline (100 mM; pH 7.0) and mixed with Ellman’s reagent (5,5′-dithiobis-(2-nitrobenzoic acid); DTNB; 1 mM) and studied compounds in DMSO (DMSO concentration was less than 0.5%) on a 96-well plate as 3 parallel assays. After reading the absorbance by the EnVision plate reader (EnVision, PerkinElmer, Inc., Waltham, MA, USA) at 412 nm, acetylthiocholine or butyrylthiocholine was added and shaken and the enzymatic activities of AChE or BChE were read at the intervals of 5 min until 30 min. The concentration of studied compounds required to inhibit the specific activity of AChE or BChE (μmol/min/mg protein) was evaluated at a concentration range of 1-800 μM and presented as a half of maximum inhibitory concentration (IC50) for each compound in each biological media at the end point (30 min). Unselective ChE inhibitor tacrine and AChE-selective inhibitor donepezil were used as a positive controls in both assays. The detailed types of inhibition of FA-derivatives 1-3 were evaluated by using 20-1000 μM concentrations of AChE substrate, acetylthiocholine, in the presence of 16, 25, or 66 μM of FA-derivatives 1-3, respectively (according to their IC50 values; Section 3.8). According to the Hanes-Woolf plots, the ratio of initial substrate concentration to the reaction velocity (μmol/min) was plotted against the substrate concentration (μM). The linear regression was used to calculate the K_m value (negative value of x-intercept) and K_max value (1/slope).

2.11. Ability of Compounds to Affect Total Amounts of mTOR and NF-κB. Enzyme-Linked Immunosorbent Assay (ELISA) kits were used (mTOR SimpleStep ELISA Kit and NFκB p65 Total SimpeStep ELISA Kit, Abcam, Cambridge, UK) to quantify mammalian (or mechanistic) target of rapamycin (mTOR) and transcription factor NF-κB amounts, respectively. The compounds (100 μM) were incubated for 48-96 hours with the cells at a density of 2 × 10^5 in 6-well plates. The control wells were treated with the solvent only (0.5% DMSO). Cells were solubilized using the provided extraction buffer with the kit, incubated on ice for 20 minutes and centrifuged at 18 000 × g for 20 min at 4°C. The supernatants were stored at -80°C till the day of the analysis. Standards and samples were then analyzed following the manufacturer’s protocol (ELISA sandwich method) and by reading the absorbance with the EnVision plate reader (EnVision, PerkinElmer, Inc., Waltham, MA, USA) at 450 nm. The results were analyzed as pmol of formed mTOR or NF-κB per mL.

2.12. Data Analysis. All the cellular studies were carried out as three biological replicates from the same cell passage. The function of LAT1 was followed between the used cell passages (7-16) with a LAT1 probe substrate, [14C]-L-leucine, and noticed to be unaltered. All statistical analyses, including Michaelis-Menten, Eadie-Hofstee, and Hanes-Woolf analyses, were performed using GraphPad Prism v. 5.03 software (GraphPad Software, San Diego, CA, USA). Statistical differences between groups were tested using one-way ANOVA, followed by Tukey’s multiple comparison test, and presented as the mean ± SD, with significant differences denoted by asterisks (*P < 0.05, **P < 0.01, and ***P < 0.001).

2.13. Ethical Statement. The experimental procedures involving animals (primary neuron and astrocyte isolation) were made in compliance with the European Commission Directives 2010/63/EU and 86/609 and approved by the Institutional Animal Care and Use Committee of University of Eastern Finland (Animal Usage Plan numbers EKS-008-2016, EKS-006-2017, and EASAI/3347/04.10.07/2015). All efforts were made to minimize the number of animals used and to minimize their suffering.

3. Results

3.1. Ability of LAT1-Utilizing Derivatives of Ferulic Acid to Bind LAT1 in Astrocytes. The ability of FA and its derivatives 1-3 to bind to LAT1 was studied in mouse primary astrocytes, from which we have recently characterized LAT1 expression and function [29]. All three derivatives of FA (1-3) were able to bind to LAT1 and inhibit the uptake of a LAT1-probe substrate, [14C]-L-leucine, at low to very low (micromolar) concentrations (Table 1). The amide derivative 1 had the greatest affinity for LAT1, and its IC50 value was lower (2.2 μM) in astrocytes as compared to previously determined values in human breast adenocarcinoma cells (MCF-7: 14.2 μM), while the opposite was observed for derivative 2 (8.9 μM vs. 1.1 μM in MCF-7). The ester analogue 3 had higher IC50 values than amide derivatives 1-2. This was most likely due to the premature bioconversion of the ester derivative to the parent drug on the cell surface, since the parent drug, FA, was not able to bind to LAT1.

3.2. Uptake of LAT1-Utilizing Derivatives of Ferulic Acid into Astrocytes via LAT1. Cellular uptake of FA and its amide derivatives 1 and 2 and ester derivative 3 into primary astrocytes was concentration-dependent (Figure S1). The uptake of FA-derivatives was 2-10 times higher than that of FA at 100 μM and 210-660 times higher at 10 μM concentrations (Table 2). The Eadie-Hofstee analysis revealed, however, that derivatives 1 and 2 were utilizing two separate transporters, the high affinity-low capacity transporter, LAT1, and another lower affinity-higher
Table 1: Ability of ferulic acid (FA) and its derivatives 1-3 to bind to LAT1 in mouse primary astrocytes presented as half maximal inhibitory concentrations (IC₅₀, μM) values of LAT1-probe substrate, [¹⁴C]-L-leucine uptake. Data are presented as the mean ± SD; n = 3-4.

| Compound          | IC₅₀ in astrocytes (μM) |
|-------------------|-------------------------|
| FA-derivative 1   | 2.15 ± 1.12             |
| FA-derivative 2   | 8.90 ± 1.25             |
| FA-derivative 3   | 28.74 ± 1.15            |
| FA                | No inhibition           |

capacity transporter (Figure S1). Curiously, FA and derivative 3 had an autoactivated Eadie-Hofstee profile. This means that FA and derivative 3 are able to induce LAT1 function or to increase its expression on the plasma membrane [30]. The affinity for LAT1-mediated uptake of FA-derivatives 1-2 was high (Kₘ values approximately 4 μM), while the affinity for the secondary transport mechanism was much lower (Kₘ values of 100-150 μM) (Table 2). Derivatives 1-2 were also more effectively transported into the astrocytes via the secondary transport mechanism (30-170 pmol/min/mg protein) than via LAT1 (8-13 pmol/min/mg protein). We have previously reported that this secondary transport mechanism is most likely the organic anion transporting polypeptides (OATPs) or the organic anion transporters (OATs), since a similar uptake pattern has also been observed in human breast cancer cells (MCF-7) and this uptake is known to be probenecid-sensitive [19, 31]. However, due to the 30-40 times higher affinity for LAT1, the role of this secondary transport mechanism in the total cellular uptake of these compounds at clinically relevant concentration (i.e., <100 μM) was considered to be minor, perhaps even nonexistent.

3.3. Intracellular Unbound Drug Concentrations of LAT1-Utilizing Derivatives of Ferulic Acid. The majority of the drug targets are localized intracellularly [8]. Therefore, it is the intracellular unbound drug concentrations that are considered to be pharmacologically relevant, and thus, it is important to evaluate drugs’ ability to interact with their final target(s). In this study, we utilized the intracellular kinetic method that has been previously proposed by Mateus et al., which combines drug’s intracellular binding and steady-state intracellular total drug concentration [25]. As a result, one can estimate drug’s unbound intracellular:extracellular ratio, which can be reported as the Kₚ,uu value. In this study, FA-derivative 1 had higher Kₚ,uu values, particularly at lower concentrations (<0.1 μM; 0.25-2.14) as compared to FA-derivative 2, which in turn had relatively similar Kₚ,uu values throughout the studied concentrations (0.04-0.08 μM; 0.83-1.74) (Figure 2). The unbound fraction of FA-derivative 1 was also slightly higher (fᵤ value of 16.26 ± 2.72) than the corresponding value of FA-derivative 2 (fᵤ value of 8.06 ± 0.54) (Table S1). This clearly demonstrates that although the cellular total uptake of FA-derivative 2 was higher than that of FA-derivative 1 (Table 2, Figure S1), the comparison of the intracellular unbound concentrations proves that the FA-derivative 1 may be a more promising drug candidate for further studies. Due to its faster metabolism, fᵤ of derivative 3 was not evaluated, and therefore, the Kₚ,uu value for derivative 3 is not reported in the present study. Unfortunately, the cellular uptake of FA was also minor, and we were not able to quantify it at lower concentrations (0.1-1 μM). Thus, it was not possible here to compare the Kₚ,uu value of FA against the FA-derivatives. However, due to its negligible cellular uptake, we can conclude that the intracellular unbound concentration of FA would also be extremely small, although there was no evidence that it was bound nonspecifically to other cell components, i.e., proteins and lipids (fᵤ value of 100%; Table S1).

3.4. Ability of Ferulic Acid and Its LAT1-Utilizing Derivatives to Inhibit Astrocyte Cell Growth. To evaluate if FA and its derivatives 1-3 can affect the viability of the primary astrocytes, the compounds were incubated at 72h with variable concentrations (5-400 μM). FA and its amide derivatives 1 and 2 as well as the ester derivative 3 did not display any antiproliferative efficacy on the astrocytes at lower concentrations but did exhibit significant anti-proliferative efficacy at concentrations above 200 μM (Figure S2). Interestingly, FA and its derivatives 1 and 2 increased the viability of these cells at low concentrations (5-100 μM). Therefore, these derivatives can be considered safe or at least nontoxic to astrocytes, when administered at clinically relevant concentrations.

3.5. Ability of Ferulic Acid and Its LAT1-Utilizing Derivatives to Inhibit Oxidative Stress and Lipid Peroxidation. The ability of FA and its derivatives 1-3 to inhibit oxidative stress and subsequent lipid peroxidation was evaluated with primary astrocytes by incubating the cells with 0.1 μg/mL LPS for 24h with and without the studied compounds and determining malondialdehyde formation (MDA) as previously described for FA [32]. As expected, 0.1 μg/mL LPS increased the lipid peroxidation in astrocytes significantly from 12.69 ± 1.47 to 23.52 ± 1.77 μmol/mg protein (185%) (Figure 3). Coincubation of LPS with the studied compounds: 50 μM tocopherol (as positive control), FA, or its LAT1-utilizing derivatives 1-3, inhibited the LPS-induced lipid peroxidation by 39–52%. Moreover, the lipid peroxidation measured in these cotreatments was comparable to the control level (11.21 ± 0.41–14.46 ± 2.28 μmol/mg protein). Curiously, the MDA levels were approximately 12% lower after derivative 2 treatment than the MDA levels in the control incubation. Nevertheless, there was no significant difference in the inhibitory activity between derivatives 1-3.

3.6. Ability of Ferulic Acid and Its LAT1-Utilizing Derivatives to Inhibit BACE1. Since FA itself has been reported to modulate directly BACE1 activity [16–18], it was also evaluated in the present study if the FA-derivatives would exert similar effects on BACE1. FA as well as all the derivatives 1-3 proved to be BACE1 inhibitors (Figure 4). The inhibitory efficacy of derivative 2 was comparable to FA at 1 and 10 μM concentrations (ca. 91–97% vs. 97%, respectively),
while the other derivatives (1 and 3) were significantly less potent than FA and derivative 2 (inhibition ca. 78–91%, respectively). Interestingly, 1 μM FA and derivative 2 inhibited BACE1 more effectively than 0.25 μM inhibitor (KTEEISEVN-Sta-VAEF-NH2) [28], provided by the kit (inhibition ca. 85%).

3.7. Ability of Ferulic Acid and Its LAT1-Utilizing Derivatives to Inhibit Peroxidase Activity. It has also been reported that FA can revert the hypoxia-induced COX-2 expression and inhibit the production of prostaglandin E2 (PGE2) within a concentration-dependent manner [33]. Therefore, the ability of FA-derivatives 1-3 to inhibit also COX enzymes was
evaluated in the present study. Both FA and derivatives 1-3 inhibited the cellular peroxidase activity of COX enzymes (Figure 5). It is noteworthy that the inhibition by FA-derivatives was significantly higher (43-54%) than with FA itself or a known COX inhibitor, diclofenac (DCF). The inhibition by DCF was only 18% in this assay, and thus, it was almost 3 times lower than with the novel FA-derivatives. However, one needs to keep in mind that the assay we used in the present study is not able to differentiate the peroxidase activity of COX enzymes from the lipoxygenases (LOX) and it has been reported that ferulic acid and its derivatives can also inhibit at least 5- and 15-LOX [34, 35]. Therefore, since FA-derivatives 1-3 had a higher ability to inhibit peroxidase activity than a clinically used COX inhibitor DCF, it is likely that these derivatives can inhibit both the production of prostaglandins and that of the leukotrienes, which broadens their multifunctional properties even wider. Nevertheless, no significant difference among FA-derivatives 1-3 was observed in this assay.

3.8. Ability of Ferulic Acid and Its LAT1-Utilizing Derivatives to Inhibit AChE/BuChE Activity. We have reported earlier that a LAT1-utilizing produg, ketoprofen, was a substrate of AChE, as measured by its IC$_{50}$ value against AChE activity in rat brain S9 fraction [36]. Furthermore, this bioconversion in rat brain S9 fraction was inhibited by donepezil, a selective inhibitor of AChE. Therefore, we wanted to evaluate if the derivatives of FA could also interact with AChE. All of the studied FA-derivatives (1-3) inhibited the activity of AChE in mouse brain S9 subcellular fraction, whereas FA itself did not have any effect (Table 3). Furthermore, amide derivatives 1 and 2 were able to inhibit AChE at micromolar concentrations, although the inhibitions were not as significant as with donepezil (IC$_{50}$ values of 16-25 µM compared to 17 nM, respectively). None of the studied compounds inhibited the activity of BuChE, although FA-derivatives 1 and 3 were able to bind to the substrate binding site of brain BuChE with very low affinity (over 220 µM IC$_{50}$ value; Table 3). The linear regression of Hanes-Woolf plots showed that all of the derivatives inhibited AChE in a mixed type manner, as the affinity for acetylthiocholine was increased: $K_m$ from 130 to 187-324 µM, respectively, while the velocity of the reaction was decreased from 2.21 nmol/min/mg protein to 2.12-2.09 nmol/min/mg protein, respectively (Figure S3 with derivatives 1 and 3). In addition to being able to bind to AChE, the bioconversion of derivative 3 in mouse brain S9 subcellular fraction was slowed down in the presence of donepezil, from a half-life of approximately 215 min to a level in which no bioconversion at all was observed. This indicates that AChE is able to slowly convert FA-derivative 3 into FA in mouse brain.

3.9. Ability of Ferulic Acid and Its LAT1-Utilizing Derivatives to Affect Total Amounts of mTOR and NF-κB. mTOR is a key regulator of the autophagy-lysosomal pathway, and thus, it has a critical role in regulating intra- and extracellular levels of Aβ in brain parenchyma [37]. Moreover, it has been reported that increased amounts of mTOR are related to elevated levels of Aβ in AD mouse brain. In addition, a natural polyphenol, resveratrol, which is structurally related to FA and an inhibitor of mTOR, has been reported to have beneficial effects against autophagy-lysosomal degradation of Aβ. It is also known that in addition to its antioxidant properties, 1 mM FA can also exert inhibitory activity on cellular mTOR signaling pathways [38]. Therefore, the inhibitory activities of FA-derivatives 1-3 (100 µM) were evaluated in this study. FA-derivatives 2 and 3 reduced the total amount of mTOR after 48 h co-incubation with the cells, along with 1 µM rapamycin or 100 µM gabapentin or metformin as positive controls (Figure 6(a)). Derivative 2 had a comparable effect (approximately 32%) to rapamycin and gabapentin (46-49%), and thus, it was significantly more potent than the other FA-derivatives. Derivative 3 reduced the total amount mTOR protein only moderately (approximately 14%), while ferulic acid itself and another positive control, metformin, did not affect to the total amount of mTOR. Moreover, FA-derivative 1 increased the amount of mTOR (approximately 28%). These discrepancies may stem from the different assay conditions, such as the concentration and time of exposure to the compounds.
from the fact that in this study we were determining the total protein amount of mTOR and not its activity, which could be measured via downstream signaling biomarkers. Thus, in the future, it would be important to clarify the exact mechanism of FA-derivatives on mTOR signaling pathways.

Transcription factor NF-κB is recognized as an important agent related to inflammation in the AD brain, since Aβ1-42 has been shown to activate NF-κB in astrocytes, which in turn has led to the increased production of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, as well as overexpression of iNOS [39]. Since mTOR signaling can also affect NF-κB via the Akt serine-threonine kinase pathway [40], it was hypothesized that FA-derivative 2, which had the greatest effect on the amount of mTOR protein (Figure 6(a)), could also alter the total NF-κB protein content. However, derivative 2 (100 μM) did not exert any effects on NF-κB levels during 24-96 h incubation (Figure 6(b)). Since the role of NF-κB in neurodegenerative diseases is not yet fully understood, it has been speculated that absolute inhibition of NF-κB may not be even a desirable property for a neuroprotective drug [39]. For example, suppression of NF-κB has been reported to increase the levels of Aβ1-42. Curiously, it has been shown recently that FA itself can inhibit the PI3K/Akt/NF-κB signaling pathway [41]. Therefore, it was concluded that unlike FA itself, FA-derivative 2 did not have any effects on NF-κB, although it was able to inhibit mTOR expression.

4. Discussion

This study shows that FA-derivatives 1-3 had many times higher uptake into the astrocytes than FA itself, especially at low concentrations, and it was primarily mediated via LAT1. Since astrocytes are known to be involved in both the inflammation and regulation of oxidative stress within the brain, they have a crucial role not only in pathophysiological changes of AD but also in other neurodegenerative diseases [20, 39, 42, 43]. Furthermore, utilizing LAT1 as a brain- and astrocyte-targeted carrier is a feasible approach to treat AD, since we have reported earlier that neither AD-induced alterations of transgenic mice nor lipopolysaccharide- (LPS-) induced inflammation changes the expression or function of LAT1 at the BBB or primary astrocytes [29, 44]. The cellular uptake was highest with derivative 3, which was able to autoactivate the transporter, followed by derivative 2, which in turn was able to utilize secondary transport mechanisms, namely, OATPs or OATs. Derivative 1 had the lowest transport rate, but had the highest selectivity towards LAT1. In contrast, the intracellular unbound drug concentrations of the FA-derivatives in astrocytes showed that derivative 1 had higher cellular bioavailability at lower concentrations. However, it needs to be remembered that the determination of Kp,uu values from cells assumes that the extracellular concentrations of FA-derivatives are completely unbound, and these may not correlate with the brain extracellular fluids [45]. Therefore, this result needs to be interpreted with caution. Furthermore, the studied cells were mouse primary astrocytes, and thus, the correlation to the corresponding human cells should be examined in the future to evaluate the full potential of these compounds in the human brain.

In addition to being potential antioxidants and preventing oxidative stress in astrocytes, FA-derivatives 1-3 also inhibited the function of BACE1, AChE, COX, and/or LOX, and one of the FA-derivatives also reduced the total amount of mTOR protein (Figure 7). Although AChE is mainly expressed in neurons and synapses, the levels of AChE have also been increased in primary astrocytic cultures treated with Aβ1-42, indicating that astrocytes may make a crucial contribution to the increased AChE activity, which can be detected in amyloid deposits in the AD brain [46]. Similarly, in addition to being present in neurons, BACE1 has also been found in activated
Astrocytes, which may have a crucial role in the accumulation of Aβ peptide levels and consequently on the neurodegeneration in AD [47]. On the other hand, COX enzymes are expressed in both glia cell types, i.e., astrocytes and microglia, and it has been reported that inhibition of COX-2 and 5-LOX in both cell types can significantly reduce the expression of multiple proinflammatory cytokines, such as TNF-α and IL-1β [48]. Together with decreased levels of eicosanoids, the reduced levels of cytokines have been reported to reduce the BACE1 activity and consequently Aβ production [49]. Therefore, the FA-derivatives that can accumulate into the astrocytes may have clinical relevance at multiple levels in AD: firstly by inhibiting AChE activity and increasing subsequently acetylcholine levels, secondly by inhibiting BACE1 and decreasing Aβ accumulation, and thirdly by reducing both oxidative stress and inflammation which can directly and indirectly inhibit BACE1 activation and the further production of Aβ. Together with increased autophagy mediated via mTOR inhibition, these compounds may have a significant potential to reduce Aβ-induced activation of astrocytes and, thus, block the self-sustaining cycle initiated by the accumulation of Aβ in AD (Figure 7) [39, 50]. However, it needs to be kept in mind that these efficacy results are only preliminary and obtained with a relatively high concentration against their target proteins. Therefore, more detailed experiments, such as in vitro compound potency studies, e.g., assessing half maximal inhibitory concentrations (IC50) and in vivo efficacy experiments with multiple doses should be carried out. In addition, the in vitro-in vivo (IVIV) correlation between these experiments should be examined to reveal the full potential of these derivatives towards each of the target proteins.

Although FA is a potential therapeutic agent to treat neurodegenerative diseases, such as AD, and also other neuroinflammatory disorders [51], its low BBB permeation and intense first pass metabolism limit its bioavailability and thus reduce its potential for treating human brain diseases [52, 53]. We have previously shown that converting FA to LAT1-utilizing derivatives 1-2 can significantly improve the BBB permeation rate, resulting in nearly 10 times greater brain exposure as measured by AUCbrain values and even higher, i.e., 30–120 times greater, Kp,brain values (AUCbrain/AUCplasma), respectively [19]. Since FA-derivatives 1 and 2 were found to be very stable in vivo, they can be considered brain-targeted multifunctional therapeutic agents, whereas derivative 3 is a prodrug and is less stable, releasing FA in vivo. However, even though derivative 3 was bioconverted quickly in mouse plasma after i.v. administration, the in vitro human data showed that the hepatic intrinsic clearance was 10 times slower than that occurring in mouse, and the bioconversion in human plasma was rather insignificant. This suggests that compound 3 may have the potential to deliver, i.e., transport and release, FA to the human brain and thus act as a prodrug of FA.

Although the prodrug approach has proven to be feasible in many cases of brain targeting as exemplified by L-dopa, a successful prodrug requires not only targeted delivery but also quantitative release of the parent drug inside the target.

**Figure 7:** A schematic presentation of the effects of FA-derivatives 1-3 on biological markers that can decrease the levels of Aβ in astrocytes in AD. Aβ: β-amyloid; AChE: acetylcholine esterase; BACE1: β-site amyloid precursor protein (APP) cleaving enzyme 1; COX: cyclooxygenases; LOX: lipoxygenases; mTOR: mammalian (or mechanistic) target for rapamycin (mTOR); NF-κB transcription factor NF-κB.

\[
x = \text{NHCH₂ (1)}
\]
\[
= \text{NH (2)}
\]
\[
= \text{O (3)}
\]

\[
\text{Oxidative stress & inflammation} \quad \rightarrow \quad \text{mTOR} \quad \rightarrow \quad \text{NF-κB} \quad \rightarrow \quad \text{BACE1} \quad \rightarrow \quad \text{Aβ}
\]

| Oxidative stress & inflammation | mTOR | NF-κB | BACE1 | Aβ |
|-------------------------------|------|-------|-------|-----|
| COX                           | LOX  | AChE  |       |     |

Aβ: β-amyloid; AChE: acetylcholine esterase; BACE1: β-site amyloid precursor protein (APP) cleaving enzyme 1; COX: cyclooxygenases; LOX: lipoxygenases; mTOR: mammalian (or mechanistic) target for rapamycin (mTOR); NF-κB: transcription factor NF-κB.
cells. Therefore, stable FA-derivatives 1 and 2, which can elicit pharmacological effects themselves, may be more promising drug candidates for further preclinical testing as compared to FA-prodrug 3. This study highlights a very important fact in drug design that converting a parent drug to transporter-utilizing derivative does not necessarily change the drug’s activity towards its target protein. In the best case scenario, the modification may even broaden the compound’s applicability and improve its potency towards other target proteins. This would be a very desirable feature, particularly in a drug intended to treat diseases like AD, in which the pathogenesis involves several distinct biochemical changes in the brain.

5. Conclusions

In conclusion, this study demonstrates that LAT1-utilizing derivatives of FA can improve the cellular uptake and thus they have better bioavailability in astrocytes, but they also have multifunctional therapeutic effects themselves. This can be especially beneficial in AD, but possibly also in the treatment of other neurodegenerative diseases or brain diseases in which neuroinflammation is involved. Moreover, this study proves that converting a parent drug into a transporter-utilizing derivative does not necessarily impair the potency of the compound towards its target protein; instead, the structural modification may broaden the spectrum of pharmacological effects elicited by the derivative.

Data Availability

The data of cellular uptake, viability and inhibition of selected biological processes used to support the findings of this study are included within the article and supplementary information file(s).

Conflicts of Interest

The authors declare no competing interest.

Authors’ Contributions

KMH, AM, and JH participated in the research design. JH, SAI, and AM conducted the experiments. KMH, AM, and SAI contributed to the new reagents or analytical tools. KMH, JH, and AM performed the data analysis. KMH, JH, and AM wrote or contributed to the writing of the manuscript. Ahmed Montaser and Johanna Huttunen contributed equally to this work.

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Supplementary Materials

Supporting Information: Figure S1: uptake of ferulic acid and its LAT1-utilizing derivatives into astrocytes via LAT1. Cellular uptake of ferulic acid (A) and its LAT1-utilizing derivatives (1-3; B-C) into primary astrocytes over a concentration range 1-100 μM (left) and the Eadie-Hofstee plots for transporter-mediated uptake mechanisms (right). The data is presented as the mean ± SD (n = 3). Table S1: intracellular unbound concentrations of ferulic acid and its LAT1-utilizing derivatives. Unbound fraction (%FA) of FA and its LAT1-utilizing derivatives 1-3. The data is presented as the mean ± SD (n = 3). Figure S2: ability of ferulic acid and its LAT1-utilizing derivatives to inhibit astrocyte cell growth. The remaining cell viability of primary astrocytes after 72 h incubation of 1-400 μM ferulic acid (A) and its LAT1-utilizing derivatives 1 (B), 2 (C), and 3 (D) presented as percentages (%) compared to the untreated cells (ctrl) (mean ± SD, n = 3-6). An asterisk denotes a statistically significant difference from the respective control (***P < 0.001, one-way ANOVA, followed by Tukey’s test).

Figure S3: ability of ferulic acid and its LAT1-utilizing derivatives to inhibit AChE/BuChE activity. Hanes-Woolf’s plots of AChE activity in the presence of 16-66 μM FA-derivatives 1 and 3 (A-B, respectively; open circles) compared to the control (filled circles). (Supplementary Materials)

References

[1] J. C. DiNunzio and R. O. Williams III, “CNS disorders—current treatment options and the prospects for advanced therapies,” Drug Development and Industrial Pharmacy, vol. 34, no. 11, pp. 1141–1167, 2008.
[2] J. Olesen, A. Gustavsson, M. Svensson et al., “The economic cost of brain disorders in Europe,” European Journal of Neuroradiology, vol. 19, no. 1, pp. 155–162, 2012.
[3] D. E. Pankevich, B. M. Altevogt, J. Dunlop, F. H. Gage, and S. E. Hyman, “Improving and accelerating drug development for nervous system disorders,” Neuron, vol. 84, no. 3, pp. 546–553, 2014.
[4] V. K. Gribkoff and L. K. Kaczmarek, “The need for new approaches in CNS drug discovery: why drugs have failed, and what can be done to improve outcomes,” Neuropharmacology, vol. 120, pp. 11–19, 2017.
[5] W. M. Pardridge, “Drug transport across the blood-brain barrier,” Journal of Cerebral Blood Flow and Metabolism, vol. 32, no. 11, pp. 1959–1972, 2012.
[6] M. Dragunow, “The adult human brain in preclinical drug development,” Nature Reviews Drug Discovery, vol. 7, no. 8, pp. 659–666, 2008.
[7] G. Lee, S. Dallas, M. Hong, and R. Bendayan, “Drug transporters in the central nervous system: brain barriers and brain parenchyma considerations,” Pharmacological Reviews, vol. 53, no. 4, pp. 569–596, 2001.
[8] J. P. Overington, B. Al-Lazikani, and A. L. Hopkins, "How many drug targets are there?", Nature Reviews Drug Discovery, vol. 5, no. 12, pp. 993–996, 2006.

[9] J. Emerit, M. Edeas, and F. Bricaire, "Neurodegenerative diseases and oxidative stress," Biomedicine & Pharmacotherapy, vol. 58, no. 1, pp. 39–46, 2004.

[10] S. Amor, L. A. Peferoen, D. Y. Vogel et al., "Inflammation in neurodegenerative diseases – an update," Immunology, vol. 142, no. 2, pp. 151–166, 2014.

[11] S. L. Albarracin, B. Stab, Z. Casas et al., "Effects of natural antioxidants in neurodegenerative disease," Nutritional Neuroscience, vol. 15, no. 1, pp. 1–9, 2012.

[12] S. E. Seidl, J. A. Santiago, H. Bilyk, and J. A. Potashkin, "The emerging role of nutrition in Parkinson’s disease," Frontiers in Aging Neuroscience, vol. 6, p. 36, 2014.

[13] B. Lin, "Polyphenols and neuroprotection against ischemia and neurodegeneration," Mini Reviews in Medicinal Chemistry, vol. 11, no. 14, pp. 1222–1238, 2011.

[14] Y. Gilgun-Sherki, E. Melamed, and D. Offen, "Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier," Neuropharmacology, vol. 40, no. 8, pp. 959–975, 2001.

[15] J. Kanski, M. Aksenova, A. Stoyanova, and D. A. Butterfield, "Ferulic acid antioxidant protection against hydroxyl and peroxy radical oxidation in synaptosomal and neuronal cell culture systems in vitro: structure-activity studies," The Journal of Nutritional Biochemistry, vol. 13, no. 5, pp. 273–281, 2002.

[16] T. Mori, N. Koyama, M. V. Guillot-Setier, J. Tan, and T. Town, "Ferulic acid is a nutraceutical β-secretase modulator that improves behavioral impairment and Alzheimer-like pathology in transgenic mice," PLoS One, vol. 8, no. 2, article e55774, 2013.

[17] J. Y. Cho, H. S. Kim, D. H. Kim, J. J. Yan, H. W. Suh, and D. K. Song, "Inhibitory effects of long-term administration of ferulic acid on astrocyte activation induced by intracerebroventricular injection of β-amyloid peptide (1-42) in mice," Progress in Neuro-Psychopharmacology & Biological Psychiatry, vol. 29, no. 6, pp. 901–907, 2005.

[18] H. S. Kim, J. Y. Cho, D. H. Kim et al., "Inhibitory effects of long-term administration of ferulic acid on microglial activation induced by intracerebroventricular injection of beta-amyloid peptide (1-42) in mice," Biological & Pharmaceutical Bulletin, vol. 27, no. 1, pp. 120–121, 2004.

[19] E. Puris, M. Gyntner, J. Huttunen, S. Auriola, and K. M. Huttunen, "L-type amino acid transporter 1 utilizing prodrugs of ferulic acid revealed structural features supporting the design of prodrugs for brain delivery," European Journal of Pharmaceutical Sciences, vol. 129, pp. 99–109, 2019.

[20] K. Gorschkov, F. Aguissanda, N. Thorne, and W. Zheng, "Astrocytes as targets for drug discovery," Drug Discovery Today, vol. 23, no. 3, pp. 673–680, 2018.

[21] R. Pihlaja, J. Koistinaho, T. Malm, H. Sikkilä, S. Vainio, and M. Koistinaho, "Transplanted astrocytes internalize deposited β-amyloid peptides in a transgenic mouse model of Alzheimer’s disease," Glia, vol. 56, no. 2, pp. 154–163, 2008.

[22] J. L. Jankowsky, D. J. Fadale, J. Anderson et al., "Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase," Human Molecular Genetics, vol. 13, no. 2, pp. 159–170, 2004.

[23] J. R. Liddell, S. Lehtonen, C. Duncan et al., "Pyrrolidine dithiocarbamate activates the Nrf2 pathway in astrocytes," Journal of Neuroinflammation, vol. 13, no. 1, p. 49, 2016.

[24] J. Huttunen, S. Peltokangas, M. Gyntner et al., "L-type amino acid transporter 1 (LAT1/Lat1)-utilizing prodrugs can improve the delivery of Drugs into neurons, astrocytes and microglia," Scientific Reports, vol. 9, no. 1, p. 12860, 2019.

[25] A. Mateus, P. Mattsson, and P. Artursson, "Rapid measurement of intracellular unbound drug concentrations," Molecular Pharmaceutics, vol. 10, no. 6, pp. 2467–2478, 2013.

[26] J. C. Kalvass and T. S. Maurer, "Influence of non-specific brain and plasma binding on CNS exposure: implications for rational drug discovery," Biopharmaceutics & Drug Disposition, vol. 23, no. 8, pp. 327–338, 2002.

[27] S. Jinno, F. Fleischer, S. Eckel, V. Schmidt, and T. Kosaka, "Spatial arrangement of microglia in the mouse hippocampus: a stereological study in comparison with astrocytes," Glia, vol. 55, no. 13, pp. 1334–1347, 2007.

[28] S. Sinha, J. P. Anderson, R. Barbour et al., "Purification and cloning of amyloid precursor protein β-secretase from human brain," Nature, vol. 402, no. 6761, pp. 537–540, 1999.

[29] M. Gyntner, E. Puris, S. Peltokangas et al., "Alzheimer’s disease phenotype or inflammatory insult does not alter function of L-type amino acid transporter 1 in mouse blood-brain barrier and primary astrocytes," Pharmaceutical Research, vol. 36, no. 1, p. 17, 2018.

[30] J. M. Hutzler and T. S. Tracy, "Atypical kinetic profiles in drug metabolism reactions," Drug Metabolism and Disposition, vol. 30, no. 4, pp. 355–362, 2002.

[31] K. M. Huttunen, J. Huttunen, I. Außerhaar, M. Gyntner, W. A. Denny, and J. A. Spicer, "L-type amino acid transporter 1 (lat1)-mediated targeted delivery of perforin inhibitors," International Journal of Pharmaceutics, vol. 498, no. 1-2, pp. 205–216, 2016.

[32] K. L. Khanduja, P. K. Avti, S. Kumar, N. Mittal, K. K. Sohi, and C. M. Pathak, "Anti-apoptotic activity of caffeic acid, ellagic acid and ferulic acid in normal human peripheral blood mononuclear cells: A Bcl-2 independent mechanism," Biochimica et Biophysica Acta, vol. 1760, no. 2, pp. 283–289, 2006.

[33] W. C. Lin, Y. F. Peng, and C. W. Hou, "Ferulic acid protects PC12 neurons against hypoxia by inhibiting the p-MAPKs and COX-2 pathways," Iranian Journal of Basic Medical Sciences, vol. 18, no. 5, pp. 478–484, 2015.

[34] K. E. Malterud and K. M. Ryland, "Inhibitors of 15-lipoxygenase from orange peel," Journal of Agricultural and Food Chemistry, vol. 48, no. 11, pp. 5576–5580, 2000.

[35] P. Morin, P.-D. St-Coeur, J. Doiron et al., "Substituted caffeic and ferulic acid phenethyl esters: synthesis, leukotrienes biosynthesis inhibition, and cytotoxic activity," Molecules, vol. 22, no. 7, p. 1124, 2017.

[36] K. M. Huttunen, "Identification of human, rat and mouse hydrolyzing enzymes bioconverting amino acid ester prodrug of ketoprofen," Bioorganic Chemistry, vol. 81, pp. 494–503, 2018.

[37] V. Galvan and M. J. Hart, "Vascular mTOR-dependent mechanisms linking the control of aging to Alzheimer’s disease," Biochimica et Biophysica Acta, vol. 1862, no. 5, pp. 992–1007, 2016.

[38] Z. Bian, N. Furuya, D. M. Zheng et al., "Ferulic acid induces mammalian target of rapamycin inactivation in cultured neurons," Oxidative Medicine and Cellular Longevity.
mammalian cells,” *Biological & Pharmaceutical Bulletin*, vol. 36, no. 1, pp. 120–124, 2013.

[39] R. E. González-Reyes, M. O. Nava-Mesa, K. Vargas-Sánchez, D. Ariza-Salamanca, and L. Mora-Muñoz, “Involvement of astrocytes in Alzheimer’s disease from a neuroinflammatory and oxidative stress perspective,” *Frontiers in Molecular Neuroscience*, vol. 10, p. 427, 2017.

[40] Y. F. Ji, L. Zhou, Y. J. Xie et al., “Upregulation of glutamate GLT-1 by mTOR-Akt-NF-κB cascade in astrocytic oxygen-glucose deprivation,” *Glia*, vol. 61, no. 12, pp. 1959–1975, 2013.

[41] G. Muthusamy, S. Gunaseelan, and N. R. Prasad, “Ferulic acid reverses P-glycoprotein-mediated multidrug resistance via inhibition of PI3K/Akt/NF-κB signaling pathway,” *The Journal of Nutritional Biochemistry*, vol. 63, pp. 62–71, 2019.

[42] E. Colombo and C. Farina, “Astrocytes: key regulators of neuroinflammation,” *Trends in Immunology*, vol. 37, no. 9, pp. 608–620, 2016.

[43] S. D. Mesquita, A. C. Ferreira, J. C. Sousa, M. Correia-Neves, N. Sousa, and F. Marques, “Insights on the pathophysiology of Alzheimer’s disease: The crosstalk between amyloid pathology, neuroinflammation and the peripheral immune system,” *Neuroscience and Biobehavioral Reviews*, vol. 68, pp. 547–562, 2016.

[44] K. S. Vellonen, J. Ihalainen, M. C. Boucau et al., “Disease-induced alterations in brain drug transporters in animal models of Alzheimer’s disease: theme: drug discovery, development and delivery in Alzheimer’s disease guest editor: Davide Brambilla,” *Pharmaceutical Research*, vol. 34, no. 12, pp. 2652–2662, 2017.

[45] A. Mateus, A. Treyer, C. Wegler, M. Karlgren, P. Mattsson, and P. Artursson, “Intracellular drug bioavailability: a new predictor of system dependent drug disposition,” *Scientific Reports*, vol. 7, no. 1, article 43047, 2017.

[46] J. Sáez-Valero, L. R. Fodero, A. R. White, C. J. Barrow, and D. H. Small, “Acetylcholinesterase is increased in mouse neuronal and astrocyte cultures after treatment with β-amyloid peptides,” *Brain Research*, vol. 965, no. 1-2, pp. 283–286, 2003.

[47] D. D. P. Tonelli, M. Mihaiovich, A. Di Cesare, F. Codazzi, F. Grohovaz, and D. Zacchetti, “Translational regulation of BACE-1 expression in neuronal and non-neuronal cells,” *Nucleic Acids Research*, vol. 32, no. 5, pp. 1808–1817, 2004.

[48] R. Pihlaja, M. Haaparanta-Solin, and J. O. Rinne, “The anti-inflammatory effects of lipoxigenase and cyclo-oxygenase inhibitors in inflammation-induced human fetal glia cells and the Aβ degradation capacity of human fetal astrocytes in an ex vivo assay,” *Frontiers in Neuroscience*, vol. 11, p. 299, 2017.

[49] W. Qin, L. Ho, P. N. Pompl et al., “Cyclooxygenase (COX)-2 and COX-1 potentiate beta-amyloid peptide generation through mechanisms that involve gamma-secretase activity,” *The Journal of Biological Chemistry*, vol. 278, no. 51, pp. 50970–50977, 2003.

[50] L. Chami and F. Checler, “BACE1 is at the crossroad of a toxic vicious cycle involving cellular stress and β-amyloid production in Alzheimer’s disease,” *Molecular Neurodegeneration*, vol. 7, no. 1, p. 52, 2012.

[51] D. Szwajgier, K. Borowiec, and K. Pustelniak, “The neuroprotective effects of phenolic acids: molecular mechanism of action,” *Nutrients*, vol. 9, no. 5, p. 477, 2017.