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Mechanistic Study on the Use of the L-Type Amino Acid Transporter 1 for Brain Intracellular Delivery of Ketoprofen via Prodrug: A Novel Approach Supporting the Development of Prodrugs for Intracellular Targets

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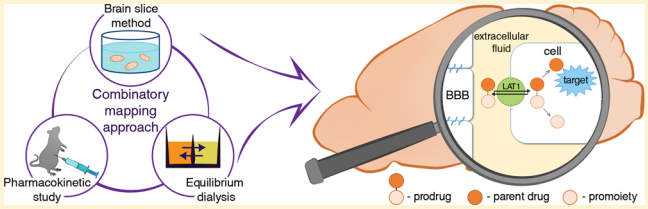
Supporting Information

ABSTRACT: L-Type amino acid transporter 1 (LAT1), selectively expressed at the blood−brain barrier (BBB) and brain parenchymal cells, mediates brain delivery of drugs and prodrugs such as L-dopa and gabapentin. Although knowledge about BBB transport of LAT1-utilizing prodrugs is available, there is a lack of quantitative information about brain intracellular delivery and influence of prodrugs on the transporter's physiological state. We studied the LAT1-mediated intrabrain distribution of a recently developed prodrug of the cyclooxygenase inhibitor ketoprofen as well as its impact on transporter protein expression and function (i.e., amino acid exchange) using brain slice method in mice and rats. The intrabrain distribution of the prodrug was 16 times higher than that of ketoprofen. LAT1 involvement in brain cellular barrier uptake of the prodrug was confirmed, reflected by a higher unbound brain intracellular compared to brain extracellular fluid concentration. The prodrug did not alter LAT1 protein expression and amino acid exchange. Integration of derived parameters with previously performed in vivo pharmacokinetic study using the Combinatory Mapping Approach allowed to estimate the brain extra- and intracellular levels of unbound ketoprofen, prodrug, and released parent drug. The overall efficiency of plasma to brain intracellular delivery of prodrug-released ketoprofen was 11 times higher than after ketoprofen dosing. In summary, this study provides quantitative information supporting the use of the LAT1-mediated prodrug approach for enhanced brain delivery of drugs with intracellular targets.



KEYWORDS: *pharmacokinetics, transporter, LAT1, prodrug, intrabrain distribution, brain slice*

INTRODUCTION

Drug delivery to the brain is an enormous challenge for the treatment of central nervous system (CNS) diseases, in particular neurodegenerative disorders. Various brain delivery approaches have been devised to overcome this problem.[1,2] Feasible brain delivery methods should be based on an understanding of the target nature and localization within the brain. Although a tremendous number of current drug targets are present in the brain extracellular space, many targets are localized in the brain parenchymal cells.[3−5] In order to interact with an intracellular target within the brain, an unbound drug needs to cross both the blood−brain barrier (BBB) and the membrane barrier of brain parenchymal cells and reach the intracellular compartment at a therapeutically effective concentration. One promising approach to deliver CNS drugs is to develop prodrugs resembling endogenous substrates of influx transporters, which are selectively expressed at the BBB and the membrane of the brain parenchymal cells. Thus, after transporter-mediated delivery of the prodrug across the BBB and cellular membrane barrier, the parent drug will be released at its target site—inside the brain parenchymal cells.

In this respect, the L-type amino acid transporter 1 (LAT1) has been successfully exploited for the brain delivery of the dopamine prodrug, L-dopa, as well as for gabapentin.[6] This transporter (Figure 1) is a pH- and Na^+-independent heterodimeric antiporter composed of light chain subunit LAT1 (SLC7A5) and heavy chain subunit CD98 (SLC3A2, also known as 4F2hc).[7] The LAT1 is responsible for the exchange of large branched and aromatic neutral amino acids (phenylalanine, leucine, histidine, tyrosine, etc.), and it plays a crucial role in the normal development and function of the CNS. The CD98 is a glycoprotein coupled with a light chain

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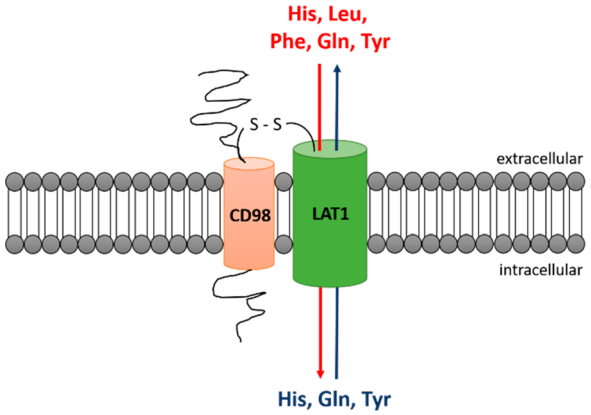

Figure 1. Representation of the transport of essential (phenylalanine, Phe; leucine, Leu; histidine, His; tyrosine, Tyr) and nonessential (glutamine, Gln) amino acids via heterodimeric transporter LAT1/CD98 across the cell membrane. LAT1 has higher affinities for L-amino acids compared to D-form as shown for Phe and Leu.[21]

that functions as a molecular chaperone localizing LAT1 in plasma membrane as well as playing a role in cell survival.[8] The expression of the LAT1 (subsequently referred to as LAT1/CD98) on both luminal and abluminal membranes of brain capillary endothelial cells as well as at the brain parenchymal cells could be exploited for brain intracellular delivery.[9−14]

Recently, we analyzed the structure−pharmacokinetic (PK) relationship of five LAT1-utilizing prodrugs of the cyclooxygenase (COX) inhibitor, ketoprofen.[15] The COX inhibitors have been reported to diminish the risk of development of Alzheimer's disease (AD),[16,17] as the COX enzyme has been postulated to be involved in AD pathogenesis.[18,19] However, since COX is abundantly expressed in peripheral organs, this means that conventional administration of COX inhibitors such as ketoprofen can be associated with systemic side effects.[20] The structure−PK relationship analysis revealed that two prodrugs with phenylalanine promoiety conjugated in *meta*- and *para*-positions to ketoprofen could cross the BBB and deliver the parent drug into the brain after i.p. injection of a single dose in wild-type mice.[15] Moreover, the distribution between liver and brain for released ketoprofen from these prodrugs was five times lower compared to that after ketoprofen dosing.[15]

However, as the COX is expressed intracellularly, it is crucial to elucidate whether and to what extent the prodrugs can cross the brain−cellular barrier and then subsequently release ketoprofen inside the brain parenchymal cells. Currently, there is only limited quantitative information about intrabrain distribution of LAT1-utilizing prodrugs. Gynther et al. provided the first evidence for intracellular brain delivery of a lysine-prodrug of ketoprofen in rats.[22] In that study, the prodrug rapidly reached the brain parenchymal cells from the brain extracellular fluid (ECF), while ketoprofen distributed mainly into the ECF. These results highlighted the potential of exploiting an LAT1-utilizing prodrug approach for brain intracellular delivery of drugs.

The assessment of brain delivery of drugs with intracellular targets via LAT1-utilizing prodrugs is a complex process since it involves monitoring the concentrations of both unbound prodrug and released parent drug in various brain compartments. Moreover, the bioconversion of the prodrug can occur in plasma and peripheral tissues; therefore, it is essential to elucidate if the release occurs in the brain. The gold standard method for "dissecting" these processes is in vivo cerebral microdialysis since this provides information about brain extracellular concentrations of unbound drugs over time. The development of a high-throughput in vitro brain slice technique has led to the introduction of the combinatory mapping approach (CMA)—an alternative method to cerebral microdialysis.[23−26] CMA integrates the data from in vitro brain slice and homogenate experiments as well as PK studies, providing a time-efficient and reliable estimation of the BBB and brain−cellular barrier transport extent of unbound drug.[23,26,27] The latter is performed by evaluating the key neuropharmacokinetic (neuroPK) parameters: unbound brain to plasma concentration ratio ($K_{p,uu,brain}$) and unbound intra- to extracellular concentration ratio ($K_{p,uu,cell}$).

The aim of the present study was to provide mechanistic insights into the intrabrain distribution of prodrugs utilizing influx transporters exemplified by LAT1 for brain delivery of drugs with intracellular targets in mice and rats. For this purpose, we used the LAT1-utilizing prodrug of ketoprofen (denoted as prodrug **1**), which previously proved capable of delivering ketoprofen into the mouse brain.[15] This is the first time that the involvement of LAT1 in intrabrain distribution of LAT1-utilizing prodrug has been investigated using the brain slice method. The effect of the prodrug on LAT1 protein expression and function was evaluated. Furthermore, we propose and discuss additional steps to the previously devised screening strategy[15] for application in the development of prodrugs utilizing influx transporters.

EXPERIMENTAL SECTION

Chemicals. Ketoprofen was obtained from Sigma-Aldrich (St. Louis, MO, USA). Prodrug of ketoprofen (prodrug **1**; (2*R*,*S*)-2-amino-3-(3-(2-(3-benzoylphenyl)propanamido)-phenyl)propanoic acid) and LAT1 inhibitor (KMH-233; (*S*)-2-amino-3-(3-((2,4-dicyano-3-(4-(2-(methylamino)-2-oxoethoxy)phenyl)benzo[4,5]imidazo[1,2-*a*]pyridin-1-yl)-carbamoyl)phenyl)propanoic acid) were synthesized based on a previously published protocol (Figure S1).[15,28] Isoflurane was purchased from Baxter Medical AB (Kista, Sweden), while 100 IU/mL heparin was from Leo Pharma AB (Malmö, Sweden). Diclofenac, which was used as the internal standard for the liquid chromatography−tandem mass spectrometry (LC−MS/MS) analysis of ketoprofen, was purchased from Sigma-Aldrich (St. Louis, MO). Internal standard peptides for quantitative proteomics analysis were purchased from JPT Peptide Technologies GmbH (Berlin, Germany). Lysyl endopeptidase (Lys-C), Protease-Max surfactant, and tosylphenylalanyl chloromethyl ketone-treated trypsin were from Promega (Madison, WI, USA). Other solvents and reagents used for intrabrain distribution study and LC−MS/MS were of high purity analytical grade or HPLC grade and were purchased from J.T. Baker (Deventer, The Netherlands), Riedel-de Haën (Seelze, Germany), Sigma (St. Louis, MO, USA), or Merck (Darmstadt, Germany). Saline solution was obtained from Braun Medical AB (Stockholm, Sweden). The water was purified using a Milli-Q Gradient system (Millipore, Bedford, Massachusetts and Millipore, Milford, USA).

Selection of the Prodrug. A *meta*-substituted phenylalanine derivative of ketoprofen (Figure S1) was selected from the previous study (denoted as prodrug **1**) based on the following characteristics.[15] The prodrug utilized LAT1 for the cellular uptake in vitro in human retinal pigmented epithelial cells (ARPE-19) crossed the BBB after in situ brain perfusion

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in adult C57BL/6 male mice. Moreover, after an i.p. injection of a single dose (25 μmol/kg) in C57BL/6 male mice (30 ± 5 g, n = 3 per compound/time point), the prodrug released ketoprofen in the brain. The ratio of areas under the concentration−time curve from time zero to 360 min for unbound ketoprofen in brain and plasma ($AUC_{u,brain}$/$AUC_{u,plasma}$) estimated using the unbound fraction in brain and plasma determined with equilibrium dialysis was 13 times higher after prodrug dosing compared to ketoprofen administration. In addition, the distribution between liver and brain of ketoprofen ($AUC_{u,liver}$/$AUC_{u,brain}$) was 5 times lower after prodrug dosing than that after ketoprofen administration.

Animals. The intrabrain distribution study using the brain slice technique was conducted in drug-naïve male Sprague−Dawley rats (250−300 g) and male C57BL/6 (20−25 g) mice in the Department of Pharmaceutical Biosciences, Uppsala University. The study protocol was approved by the Animal Ethics Committee (C189/14). The animals were obtained from Taconic (Lille Skensved, Denmark). The animals (21 rats; 21 mice) were group-housed in stainless steel cages at an ambient temperature of 20−22 °C with a humidity-controlled environment under a 12 h light/dark cycle. The animals had *ad libitum* access to food and tap water.

Intrabrain Distribution Study Using an in Vitro Brain Slice Method. *Assessment of the Unbound Volume of Distribution in the Brain.* The study of intrabrain distribution of ketoprofen and its prodrug in the brain of mice (n = 3 per study group) or rats (n = 3 per study group) was performed with the previously published brain slice protocol with small modifications.[23,25] Briefly, six and ten 300 μm coronal slices were prepared from each rat or mouse brain, respectively, using a vibrating blade microtome Leica VT1200 (Leica Microsystems AB, Sweden). The slices were placed in an 80 mm diameter beaker, containing 15 mL (for rats) or 12 mL (for mice) of preoxygenated artificial extracellular fluid (aECF) with either 0.2 μM ketoprofen or 0.2 μM prodrug. The aECF used to maintain the brain slices contained 129 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 1.4 mM $CaCl_2$, 10 mM glucose, and 25 mM HEPES, and 0.4 mM ascorbic acid. At room temperature, the aECF pH was adjusted to 7.6. The beakers, covered by a lid made of a Teflon fluorinated ethylene-propylene film (Teflon FEP film 50 Å, 12.7 μm thickness), were incubated in a shaker (MaxQ4450, Thermo Fisher Scientific, NinoLab, Sweden) at 37 °C, 45 rpm, for 5 h under a constant flow of oxygen in the incubation chamber to preserve slice viability. When studying the dynamics of uptake of the compounds, 200 μL of aECF buffer was collected at the beginning of the experiment, and at 0.5, 1, 1.5, 2, 3, 4, and 5 h after incubation in Eppendorf tubes, containing 200 μL of blank rat or mouse brain homogenate in aECF (1:4, w/v) without the investigated compounds. The latter was performed to ensure matching of the matrix composition among all samples in the subsequent analysis. After 5 h incubation, the excess buffer was carefully removed from the brain slices with a filter paper. The slices were weighed and homogenized in aECF (1:9, w/v) individually with an ultrasonic processor (VCX-130, Sonics, Chemical Instruments AB, Sweden). All samples were stored at −20 °C until LC−MS/MS analysis as described below. The viability of the brain slices was studied via a dynamic pH measurement and assessment of lactate dehydrogenase activity release using a cytotoxicity detection kit (Roche Diagnostics GmbH, Germany).

In the assessment of intrabrain distribution, we determined $V_{u,brain}$(mL/g brain), a neuroPK parameter describing drug intrabrain distribution driven by various processes including nonspecific and specific brain tissue binding, active transport across cellular membrane, and pH partitioning of the drug. The evaluation assumes that at equilibrium the concentration of the compounds in the virtually protein-free aECF in the beaker is equal to the brain interstitial fluid or ECF unbound concentration in the brain slice. In context of physiological volumes of brain fluids, $V_{u,brain}$ > 0.8 mL/g brain represents the intracellular distribution and/or tissue binding, and/or distribution to subcellular organelles, while $V_{u,brain}$ < 0.8 mL/g brain represents the distribution to the brain ECF with minimal protein or membrane binding.[25] $V_{u,brain}$ was calculated using eq 1, as described:[23,25]

$$V_{u,brain} = \frac{A_{brain} - V_i C_{buffer}}{C_{buffer}(1 - V_i)} \quad (1)$$

where A_{brain} (nmol/g brain) is the drug amount in the brain slice, C_{buffer} is the aECF concentration of compounds after 5 h incubation; V_i (mL/g brain), the volume of the surrounding brain slices layer of aECF, equal to 0.094 mL/g brain, was used based on previous studies with marker [^{14}C]-inulin.[23]

As a direct measurement of intracellular unbound concentrations is currently not feasible, the intracellular-to-extracellular partitioning coefficient ($K_{p,uu,cell}$) for unbound ketoprofen and prodrug was estimated using eq 2 introduced by Friden et al.[27] Herein, the eq 2 is used for $K_{p,uu,cell}$ calculation, while theoretical and mathematical details can be found in Friden et al.[27] $K_{p,uu,cell}$ describes the distribution of the unbound compound between the brain intracellular fluid (ICF) and extracellular compartment, representing an average ratio for all types of parenchymal cells at steady-state. The estimation of the parameter assumes that the compounds are unbound in brain ECF. $K_{p,uu,cell} \geq 1$ represents the intracellular distribution of the compounds, while $K_{p,uu,cell} < 1$ reflects the domination of the extracellular distribution.

$$K_{p,uu,cell} = \frac{V_{u,brain} - V_{brain,ECF}}{V_{ICF} V_{u,ICF}} \quad (2)$$

where $V_{u,brain}$ is the unbound volume of distribution; $V_{brain,ECF}$ is the physiological fractional volume of the brain ECF, estimated to be 0.2 mL/g brain;[29] V_{ICF} is the physiological fractional volume of the intracellular compartment, estimated to be 0.8 mL/g brain;[30] $V_{u,ICF}$ is the distribution volume of unbound drug in the ICF. It characterizes the drug intracellular binding, calculated using eq 3:[27]

$$V_{u,ICF} = 1 + \frac{D}{V_{ICF}}\left(\frac{1}{f_{u,homogenate}} - 1\right) \quad (3)$$

where $f_{u,homogenate}$ is an unbound fraction of the investigated compound in diluted brain homogenate determined by equilibrium dialysis described in section Equilibrium Dialysis.

Evaluation of LAT1 Contribution to Intrabrain Distribution of Prodrug. The intrabrain distribution of the prodrug in mice and rats (n = 3 per species) was studied in the presence of the recently developed selective LAT1 inhibitor (KMH-233).[28] The brain slices were preincubated in aECF containing 800 nM LAT1-inhibitor for 30 min before adding 200 nM prodrug into the beaker. Subsequently, aECF samples were collected at 0.5, 1, 1.5, 2, 3, 4, and 4.5 h after adding the

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prodrug. The brain slices were collected at 4.5 h after adding the prodrug and stored at −20 °C prior to the LC−MS/MS analysis, as the optimal time for the slice study is 5 h. Consequently, $V_{u,brain}$ and $K_{p,uu,cell}$ for the prodrug were calculated according to eqs 1 and 2. We assumed that the LAT1 inhibitor would have no impact on the brain tissue binding properties of the prodrug. Therefore, in the calculations, the same $f_{u,homogenate}$ of the prodrug was used, as that measured without LAT1 inhibitor.

Profiling of Transporter Protein Expression and Amino Acids Levels in Brain Slice Study. In the quantitative protein expression and amino acid analysis, the brain slices from rats (n = 3 per experiment) and mice (n = 3 per experiment), which had been incubated for 5 h in aECF with or without 200 nM prodrug or 800 nM LAT1 inhibitor were collected, weighed, and stored in Eppendorf tubes at −80 °C until LC−MS/MS analysis. The nonincubated control brain slices from mice (n = 3) and rats (n = 3) were collected and stored in Eppendorf tubes at −80 °C until LC−MS/MS analysis. During the experiment, 500 μL (rat slices) and 250 μL (mouse slices) of aECF buffer were collected before incubation, after 1 and 5 h of incubation into Eppendorf tubes for amino acid analysis. Samples were stored at −80 °C until LC−MS/MS analysis (section Bioanalysis of Samples).

Equilibrium Dialysis. The homogenate method was used to determine the unbound fraction of the prodrug and LAT1 inhibitor in rat brain homogenate in phosphate-buffered saline (PBS), pH 7.4 (1:4, w/v) using an equilibrium dialysis technique. Briefly, the investigated compounds were added to the brain homogenate at a final concentration of 10 μM, vortexed, and placed (100 μL) in the sample chamber of the rapid equilibrium dialysis (RED) devices (Thermo Scientific, Woburn, MA). PBS (350 μL) was added to the corresponding buffer chamber. The homogenate was dialyzed in a shaker at 200 rpm and 37 °C for 5 h. Samples from both chambers were taken, and an equal volume of blank homogenate or PBS was added to ensure analytical identity. The unbound fraction ($f_{u,brain}$) of prodrug and LAT1 inhibitor in the brain was calculated using eq 4:[31]

$$f_{u,brain} = \frac{f_{u,homogenate}}{D - (D-1)f_{u,homogenate}} \quad (4)$$

where D is the dilution factor of tissue (D = 5) and $f_{u,homogenate}$ is the ratio of compound concentration measured in the tissue sample and buffer within the equilibrium dialysis assay. The unbound fraction of ketoprofen, the prodrug, and LAT1 inhibitor in plasma ($f_{u,plasma}$) and mouse brain as well as the unbound fraction of ketoprofen in rat brain were determined using a similar method and have been reported previously.[15,22,28]

It is considered that $f_{u,brain}$ measured by the brain homogenate method is equal to $1/V_{u,brain}$, if the drug distribution within the cell is mainly driven by nonspecific and specific binding, and not by active uptake or efflux.[32] However, when active processes are involved, $f_{u,brain}$ may under- or overestimate the value of $V_{u,brain}$, which may contribute to errors in the subsequent assessment of $K_{p,uu,brain}$.

Evaluation of NeuroPK Parameters. The CMA was used to determine the parameters describing the BBB transport and intrabrain distribution of ketoprofen and the prodrug in mice.[15,26] In addition, we estimated the contribution of ketoprofen released in plasma to the brain intracellular levels of unbound released ketoprofen after dosing of the prodrug. In our previous PK study after an i.p. injection of a single dose (25 μmol/kg) of either ketoprofen or prodrug in mice, $AUC_{u,brain}$ and $AUC_{u,plasma}$ were estimated using the unbound fraction in brain and plasma determined with equilibrium dialysis.[15] However, $AUC_{u,brain}$ determined with brain homogenate does not provide a comprehensive picture of the intrabrain distribution of the prodrug and release of ketoprofen. In the current study, the extent of the brain delivery and intrabrain distribution of ketoprofen and prodrug in mice was estimated with the more reliable brain slice method via the evaluation of $V_{u,brain}$ combined with the previously reported PK parameters.[15]

Extent of Brain Delivery of Ketoprofen and Prodrug. The extent of delivery of investigated compounds across the BBB was evaluated by calculating the ratio of total concentrations in brain and plasma ($K_{p,brain}$, eq 5), and the unbound brain ECF to plasma concentration ratio ($K_{p,uu,brain}$, eq 6):

$$K_{p,brain} = \frac{AUC_{total,brain}}{AUC_{total,plasma}} \quad (5)$$

$$K_{p,uu,brain} = \frac{K_{p,brain}}{V_{u,brain}f_{u,plasma}} \quad (6)$$

where $AUC_{total,brain}$ and $AUC_{total,plasma}$ are areas under the total concentration−time curve from time zero to 360 min in brain and plasma, respectively, measured after the 25 μmol/kg single dose of either ketoprofen or prodrug i.p. in mice.[15] The brain total concentration was corrected for the residual blood concentration using the method described by Murakami et al.[33] The $f_{u,plasma}$ is the unbound fraction of compound in plasma.

$K_{p,uu,brain}$ has the advantage that it provides quantitative information about the net flux of drug across the BBB, including active and passive transport without the impact of plasma and brain tissue binding. A value of $K_{p,uu,brain} < 1$ indicates that there is active efflux of the drug from the brain, while $K_{p,uu,brain} > 1$ is evidence of an active influx of the drug into the brain.

Estimation of Intracellular and Extracellular Distribution of Ketoprofen and Prodrug. After crossing the BBB, the unbound compound enters the brain ECF, and this is followed by its distribution to the brain parenchymal cells. The $AUC_{u,brainECF}$ for unbound ketoprofen and prodrug was calculated according to eq 7:

$$AUC_{u,brainECF} = \frac{AUC_{total,brain}}{V_{u,brain}} \quad (7)$$

The $AUC_{u,brainICF}$ for unbound ketoprofen and prodrug was estimated using eq 8, derived from eqs 2 and 7:

$$AUC_{u,brainICF} = \frac{AUC_{total,brain}(V_{u,brain} - V_{brainECF})}{V_{ICF}V_{u,ICF}V_{u,brain}} \quad (8)$$

Estimation of Intracellular and Extracellular Distribution of Ketoprofen Released in Plasma. The $AUC_{total,brain}$ of ketoprofen includes both ketoprofen released in the brain and ketoprofen that has entered from plasma. Therefore, eqs 9 and 10 were used for the estimation of $AUC_{u,brainECF}$ and $AUC_{u,brainICF}$ of unbound ketoprofen, respectively, which was released in plasma from the prodrug and further distributed into the brain ECF after crossing the BBB:

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$$AUC_{u,brainECF} = K_{p,uu,brain} AUC_{total,plasma} f_{u,plasma} \quad (9)$$

$$AUC_{u,brainICF} = K_{p,uu,cell} AUC_{u,brainECF} \quad (10)$$

where $AUC_{total,plasma}$ of released ketoprofen had been quantified in the previous PK study after prodrug dosing.[15] We assumed that ketoprofen released in plasma from the prodrug would follow similar kinetics as after ketoprofen dosing. Therefore, the same values for $K_{p,uu,brain}$ and $K_{p,uu,cell}$ as those calculated for ketoprofen administration were used.

Estimation of Intracellular Release of Ketoprofen. The main assumption of the prodrug approach is that bioconversion to the parent drug occurs in the intracellular compartment including brain parenchymal cells. $AUC_{total,brain}$ of ketoprofen quantified after prodrug dosing does not allow for the estimation of the site of bioconversion. Therefore, a general approach described by eqs 7 and 8 was used to estimate $AUC_{u,brainECF}$ and $AUC_{u,brainICF}$, respectively, for ketoprofen released from the prodrug in the brain ECF and ICF. The estimated $AUC_{u,brainECF}$ and $AUC_{u,brainICF}$ included both ketoprofen released in the brain and delivered from plasma after prodrug dosing. Therefore, $AUC_{u,brainICF}$ of ketoprofen distributed to the ICF from plasma (section Estimation of Intracellular and Extracellular Distribution of Ketoprofen Released in Plasma) was subtracted from $AUC_{u,brainICF}$ including the ketoprofen released in plasma and brain to estimate $AUC_{u,brainICF}$ of unbound ketoprofen released in the intracellular compartment of the brain. Thus, the estimated $AUC_{u,brainICF}$ of ketoprofen released in the brain intracellular compartment was calculated using eq 11:

$$AUC_{u,brainICF} = \frac{AUC_{total,brain}(V_{u,brain} - V_{brainECF})}{V_{ICF} V_{u,ICF} V_{u,brain}} - AU$$

$$C_{u,brainICF(plasma\text{-}released\ ketoprofen)} \quad (11)$$

$AUC_{total,brain}$ of ketoprofen released in the whole brain after prodrug dosing had been quantified previously,[15] $AU-C_{u,brain\ (plasma\text{-}released\ ketoprofen)}$ is unbound ketoprofen, which has been released in plasma from the prodrug and further distributed into the brain ECF after crossing the BBB followed by entering the brain ICF (calculated in section Estimation of Intracellular and Extracellular Distribution of Ketoprofen Released in Plasma).

This approach is based on the fact that unbound ketoprofen achieves an equilibrium between brain ECF and ICF. It is important to remember that if the bioconversion occurs inside the parenchymal cells, then the process will be more complex, and equilibrium is only reached when the rate of elimination from the brain ICF, or binding or distribution to cell organelles of released unbound ketoprofen, is equal to the bioconversion rate inside the cells. However, as this cannot be proved, this approach may underestimate $AUC_{u,brainICF}$ values and overestimate $AUC_{u,brainECF}$ values.

Bioanalysis of Samples. *Quantification of Ketoprofen, Prodrug, and LAT1 Inhibitor.* In the quantification of ketoprofen, prodrug, and the LAT1 inhibitor in experimental samples, an aliquot of 100 μL of the homogenates from brain slices or equilibrium dialysis samples was precipitated by adding 300 μL of acetonitrile containing the internal standard, diclofenac (50 nM). The samples were vortexed and centrifuged for 10 min at 14000 × *g* at 4 °C. Consequently, 100 μL of supernatant was mixed with 100 μL of water before the LC−MS/MS analysis described below. The calibration curve standards and blank samples were prepared in a similar way in the respective control matrices. The accuracy, precision, linearity, and lower limits of quantification (LLOQ) for the LC−MS/MS methods were tested according to the European Medicines Agency (EMA) guideline.[34] The calibration curves were considered acceptable, when accuracy and precision were >20% for the LLOQ and >15% for the whole measurement range.

The LC−MS/MS quantification of ketoprofen, prodrug, and LAT1 inhibitor was performed using the previously described methods.[15,28] Briefly, the compounds were separated in an Agilent 1200 Series Rapid Resolution LC System (Agilent Technologies, Waldbronn, Germany) coupled with Agilent 6410 Triple Quadrupole Mass Spectrometer with an electrospray ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA). Poroshell 120 EC-C-18 column (50 mm × 2.1 mm, 2.7 μm) was used. The aqueous eluent phase contained 0.1% formic acid in water, while the organic phase consisted of pure acetonitrile. The gradient for mobile phase in the analysis of ketoprofen and prodrug was as follows: (0−0.5 min) 20% organic phase, (0.5−1 min) 20−80% organic phase, (1−4.5 min) 80% organic phase followed by 3.5 min equilibration of the column with a flow rate of 0.4 mL/min. A gradient elution for the LAT1 inhibitor was as follows: (0−1 min) 20% organic phase, (1−3 min) 20−90% organic phase followed by a 3 min column equilibration with a flow rate of 0.3 mL/min. Mass spectrometric detection was performed with multiple reaction monitoring (MRM) in the positive mode with the following transitions: 255 → 209 for ketoprofen; 417 → 134.8 for the prodrug; 588 → 191.6 for the LAT1 inhibitor; and 296.1 → 250 for diclofenac. Data was acquired using the software Agilent MassHunter Workstation Acquisition (Agilent Technologies, Data Acquisition for Triple Quad., B.03.01) and processed with Quantitative Analysis (B.04.00) software. All samples were within the identified linear range of individual calibration curves (determination coefficients $R^2 > 0.980$). The LLOQ of ketoprofen, LAT1 inhibitor, and prodrug were 5, 10, and 1 nM, respectively.

Protein Quantification Analysis. The protein expression levels of LAT1, CD98, glucose transporter type 1 (GLUT1), and Na^+/K^+-ATPase in crude membrane fractions of mouse and rat brain slices were quantified using multiplexed MRM analysis according to the protocol described by Uchida et al.[35] GLUT1 was used as a marker, indicating changes in cellular energy metabolism during the experiments. First, the crude membrane fractions were isolated using ProteoExtract Subcellular Proteome Extraction Kit (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. The aliquots (50 μg of protein) were solubilized in 7 M guanidine hydrochloride, 500 mM Tris−HCl (pH 8.5), and 10 mM EDTA. The proteins were reduced with dithiothreitol and *S*-carbamoylmethylated with iodoacetamide. Subsequently, proteins were precipitated with methanol and chloroform. The precipitates were dissolved by addition of 6 M urea in 0.1 M Tris−HCl (pH 8.5) followed by a 5-fold dilution with 0.1 M Tris−HCl (pH 8.5), which was spiked with a mixture of internal standard peptides. This step was followed by the addition of Lys-C and Protease-Max and incubation at room temperature for 3 h. Finally, tosylphenylalanyl chloromethyl ketone-treated trypsin was added for tryptic digestion of the samples (enzyme/substrate ratio of 1:100), which were incubated at 37 °C for 16 h. Formic acid in water 20% (v/v) was used to acidify the samples, followed by centrifugation

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Table 1. NeuroPK Parameters Characterizing Intrabrain Distribution in Mice and Rats[a]

	unit	ketoprofen	prodrug	prodrug after LAT1 inhibitor	LAT1 inhibitor
Mice					
$f_{u,brain}$		0.15 ± 0.018[b]	0.067 ± 0.012[b]	0.067 ± 0.011[c]	0.01[b]
$V_{u,brain}$	mL/g brain	1.47 ± 0.403	24.3 ± 6.07	16.9 ± 1.11	4.71 ± 0.848
$K_{p,uu,cell}$		0.197	1.46	1.01	0.0450
A_{brain}	nmol/g brain	241 ± 10.3	2800 ± 207	1450 ± 43.8	3180 ± 227
Rat					
$f_{u,brain}$		0.24[b]	0.030 ± 0.004	0.030 ± 0.004[c]	0.015 ± 0.001
$V_{u,brain}$	mL/g brain	1.84 ± 0.162	31.4 ± 2.73	22.2 ± 3.44	5.14 ± 0.446
$K_{p,uu,cell}$		0.240	1.87	1.32	0.0490
A_{brain}	nmol/g brain	247 ± 5.60	3050 ± 144	1850 ± 37.8	3280 ± 66.6

[a]Fraction of unbound drug ($f_{u,brain}$), unbound volume of distribution in brain ($V_{u,brain}$), unbound intra-to-extracellular concentration ratio ($K_{p,uu,cell}$), and the amount of the compound in the brain slice (A_{brain}) for ketoprofen or prodrug (200 nM) with or without 30 min pre-incubation with the LAT1 inhibitor (800 nM) in mouse and rat brain slices. Data are presented as mean ± SD. [b]Value was measured and reported previously.[15,22,28] [c]We suggested that the LAT1 inhibitor would not affect brain tissue binding properties of prodrug. Hence, $f_{u,brain}$ of the prodrug with and without the LAT1 inhibitor was assumed to be the same.

at 14000 × *g* for 5 min at 4 °C. The supernatants were subjected to LC−MS/MS analysis.

The LC−MS/MS analysis was conducted by coupling using an Agilent 1290 Infinity LC (Agilent Technologies, Waldbronn, Germany) system to an Agilent 6495 Triple Quadrupole Mass Spectrometer equipped with an ESI source (Agilent Technologies, Palo Alto, CA, USA). The HPLC method used for separation and elution of peptides has been described previously using AdvanceBio Peptide Map column (2.1 × 250 mm, 2.7 μm).[36] The eluted peptides were simultaneously detected using the positive ion MRM mode. The dwell time was 20 ms per transition. The source temperature was 210 °C with drying gas at a flow rate of 16 L/min. The nebulizer pressure was 45 psi and MS capillary voltage was 3 kV. The quantitation of the target protein was based on one unique peptide selected according to the in silico peptide selection criteria and previous reports.[35,37] Three or four MRM transitions for each specific peptide related to high intensity fragment ions were selected for quantification of a stable isotope-labeled peptide and the unlabeled investigated peptide (Table S1). Data were acquired using the Agilent MassHunter Workstation Acquisition software (Agilent Technologies, Data Acquisition for Triple Quad., version B.03.01) and processed with Skyline software (version 4.1). The expression levels of target proteins in crude membranes of mouse and rat brain slices were expressed as absolute values and as a ratio normalized to the expression level of Na^+/K^+-ATPase, a membrane marker, to allow comparison of protein expression between samples.[38]

Analysis of Amino Acids in Brain Slices and aECF. In the quantification of amino acids in the experimental samples, brain slices (10−20 mg) were homogenized (1:20, w/v) in 80% methanol in water. The samples were vortexed and centrifuged for 10 min at 14000 × *g* at 4 °C. Then, 200 μL of supernatant was used for the analysis described below. The quantifications of L-phenylalanine (L-Phe) and L-glutamine (L-Gln) (analysis 1) in brain slices and aECF were carried out using the previously reported method with the UHPLC-QTOF-MS System (Agilent Technologies 1290 LC, 6540 MS, Agilent Technologies, Santa Clara, CA, USA).[39] In the quantification of L-tyrosine (L-Tyr), L-histidine (L-His), and L-leucine (L-Leu) (analysis 2), an Agilent 1200 Series Rapid Resolution LC System (Agilent Technologies, Waldbronn, Germany) was used coupled with Agilent 6410 Triple Quadrupole Mass Spectrometer with an electrospray ionization source (Agilent Technologies, Palo Alto, CA, USA). Hydrophilic interaction chromatography (HILIC) combined with positive mode ESI was performed in both analyses. An Acquity UPLC BEH Amide column (100 mm × 2.1 mm, 1.7 μm; Waters Corporation, Milford, MA, USA) was used with a flow rate 0.6 mL/min in analysis 1 and 0.3 mL/min in analysis 2. The eluents A and B were 50% and 90% ACN, respectively, both containing 20 mM of pH 3 ammonium formate. In analysis 1, the gradient was as follows: (0−2.5 min) 100% B; (2.5−10 min) 100% B → 0% B; (10−10.01 min) 0% B → 100% B; (10.01−12.5 min) 100% B. In the data acquisition, the mass range was 20−1600 atomic mass unit (amu) with a scan speed of 600 ms. In the automatic MS/MS analyses, quadrupole isolation width was 1.3 amu. The amino acids were verified based on their fragmentation and retention times relevant to the standard compounds. In the high-resolution MS quantitation, the following ions were selected: 166.0871 for L-Phe and 146.9801 for L-Gln. Data acquisition was conducted with MassHunter Acquisition B.04.00 (Agilent Technologies, Santa Clara, CA, USA) and processed with Quantitative Analysis (B.07.00). In analysis 2, the gradient was as follows: (0−2.5 min) 95% B; (2.5−7.5 min) 95% B → 50% B; (7.5−8 min) 50% B → 95% B; (8−12 min) 95% B. Mass spectrometric detection was performed with MRM in the positive mode with the following transitions: 182 → 164.9 for L-Tyr; 156 → 110 for L-His; 132.1 → 86.1 for L-Leu. Data was acquired using the software Agilent MassHunter Workstation Acquisition (Agilent Technologies, Data Acquisition for Triple Quad., B.03.01) and processed with Quantitative Analysis (B.04.00) software. The methods were determined to possess acceptable (≤15% of the relative standard deviation) precision and accuracy. The calibration curve showed good linearity (determination coefficients R^2 > 0.980). The LLOQ for of L-Phe, L-Gln, L-Tyr, L-His, and L-Leu ranged between 0.05 and 0.1 nM, respectively.

Data Analysis. The data are presented as median (range) for protein expression levels and as mean ± SD for $V_{u,brain}$, A_{brain}, and $f_{u,brain/plasma}$. Data analysis was done using GraphPad Prism, version 5.03 (GraphPad Software, San Diego, CA).

RESULTS

Evaluation of Intrabrain Distribution Using the Brain Slice Method. The results from the intrabrain distribution

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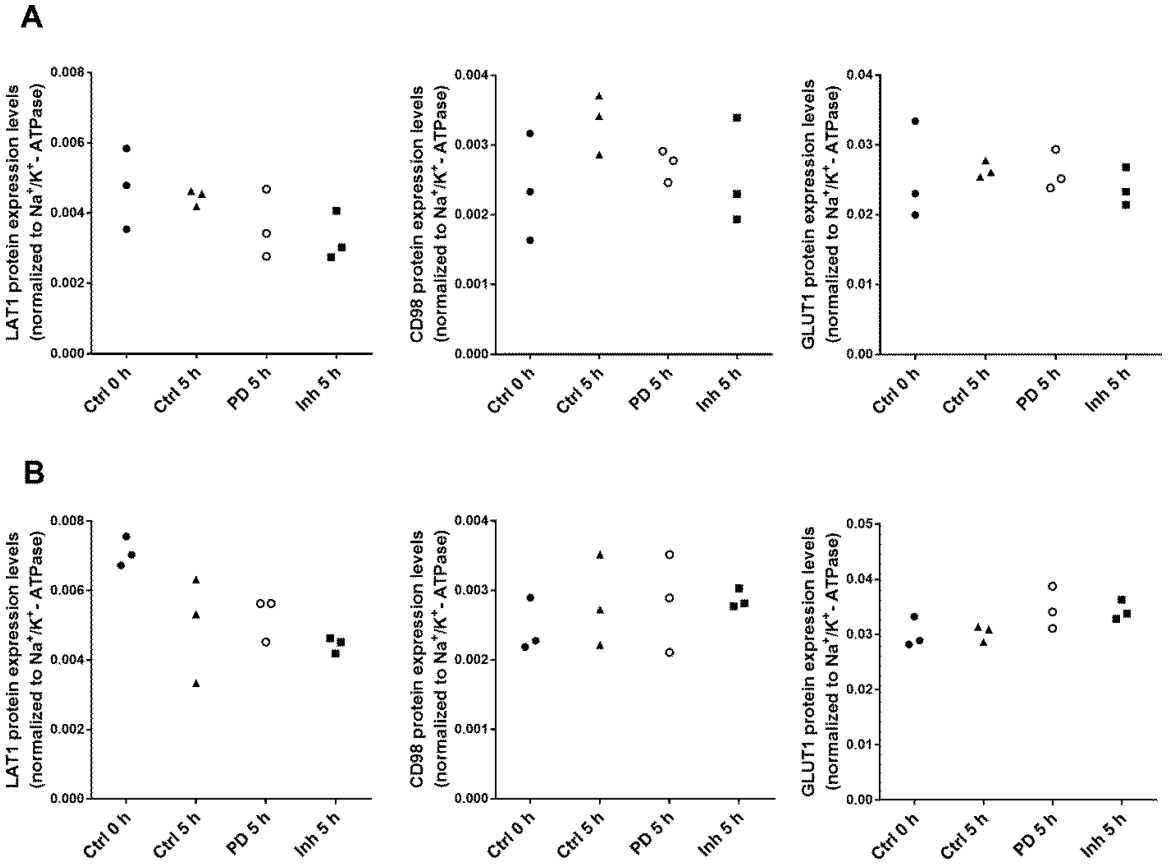

Figure 2. LAT1, CD98, and GLUT1 protein expression levels in crude membranes of mouse (A) and rat (B) brain slices at baseline (●, Ctrl 0 h), after 5 h of incubation in aECF (▲, Ctrl 5 h), and after 5 h incubation in aECF containing 200 nM prodrug (○, PD 5 h) or 800 nM LAT1 inhibitor (■, Inh 5 h). The protein expression levels were normalized to that of the membrane marker, Na^+/K^+-ATPase. The data is presented for each experiment illustrating values from individual animals (n = 3 per group).

study demonstrated that the prodrug had more than 16 times higher value of $V_{u,brain}$ than ketoprofen itself in both mice and rats (Table 1). Both compounds reached equilibrium between aECF and brain slices within 5 h (Figure S2). The incubation of brain slices with the selective LAT1 inhibitor decreased $V_{u,brain}$ of the prodrug by approximately 30% in both mice and rats.

No released ketoprofen from the prodrug was detected in either brain slices or aECF during the experiments. The $f_{u,brain}$ of ketoprofen (0.15 and 0.24) was higher than that of the prodrug (0.067 and 0.030) in both mice and rats, respectively. The $K_{p,uu,cell}$ displayed a higher intracellular distribution of the prodrug (≥1) in comparison to the parent drug (<1) in both species. Moreover, there was a subtle species difference in $K_{p,uu,cell}$ and $V_{u,brain}$ of both ketoprofen and prodrug with higher levels observed in rats, whereas the amounts of ketoprofen and prodrug in brain slices were similar in both species (Table 1). The $K_{p,uu,cell}$ of the prodrug decreased after incubation with the LAT1 inhibitor. The inhibitor itself reached equilibrium within 5 h (Figure S2) and demonstrated around 5-fold lower $V_{u,brain}$ than the prodrug in both species.

Transporter Expression in Brain Slices. The study demonstrated that a 5 h incubation with or without prodrug or the LAT1 inhibitor did not alter the crude membrane protein expression of LAT1, but CD98 normalized to the expression levels of Na^+/K^+ ATPase in mouse and rat slices (Figure 2). There was a trend toward a decrease in normalized LAT1 protein expression levels in rat slices after incubation for 5 h, which was more pronounced in the LAT1 inhibitor coincubation group. The LAT1 absolute expression levels were not species-dependent and ranged from 0.37 to 0.82 fmol/μg in mouse slices vs 0.58 to 1.27 fmol/μg in rat slices (Table S2). CD98 had lower expression than LAT1 with absolute levels of 0.19−0.58 fmol/μg in mice and 0.28−1.13 fmol/μg in rats. GLUT1 protein expression levels remained the same during the experiments, evidence that there had been no disturbance in energy metabolism in brain slices. GLUT1 expression in brain crude membrane was more than 3 and 10 times higher than that of LAT1 and CD98, respectively (Figure 2).

Effects of Prodrug and LAT1 Inhibitor on Amino Acid Transport. The potential of the prodrug and LAT1 inhibitor to interfere with the transport of the main endogenous amino acids, substrates of LAT1, such as nonessential glutamine (L-Gln) and essential amino acids (L-Phe, L-Leu, L-His, L-Tyr), was evaluated in mouse and rat brain slices (Figure 3). It was observed that concentrations of L-His, L-Tyr, and L-Phe in mouse and rat brain slices remained the same during the 5 h incubation with or without the prodrug or the LAT1 inhibitor. However, the incubation with or without the prodrug or the LAT1 inhibitor reduced the concentrations of L-Gln from 8.7−11.6 nmol/g brain to 3.2−4.7 nmol/g brain in mouse slices and from 6.5−8.6 nmol/g brain to 1.7−2.5 nmol/g brain in rat slices. Similarly, L-Leu concentrations in mouse and rat slices were reduced from 10.3−12.9 nmol/g brain to 6.1−9.4 nmol/g brain and from 21.8−30.4 nmol/g brain to 8.5−13.3 nmol/g

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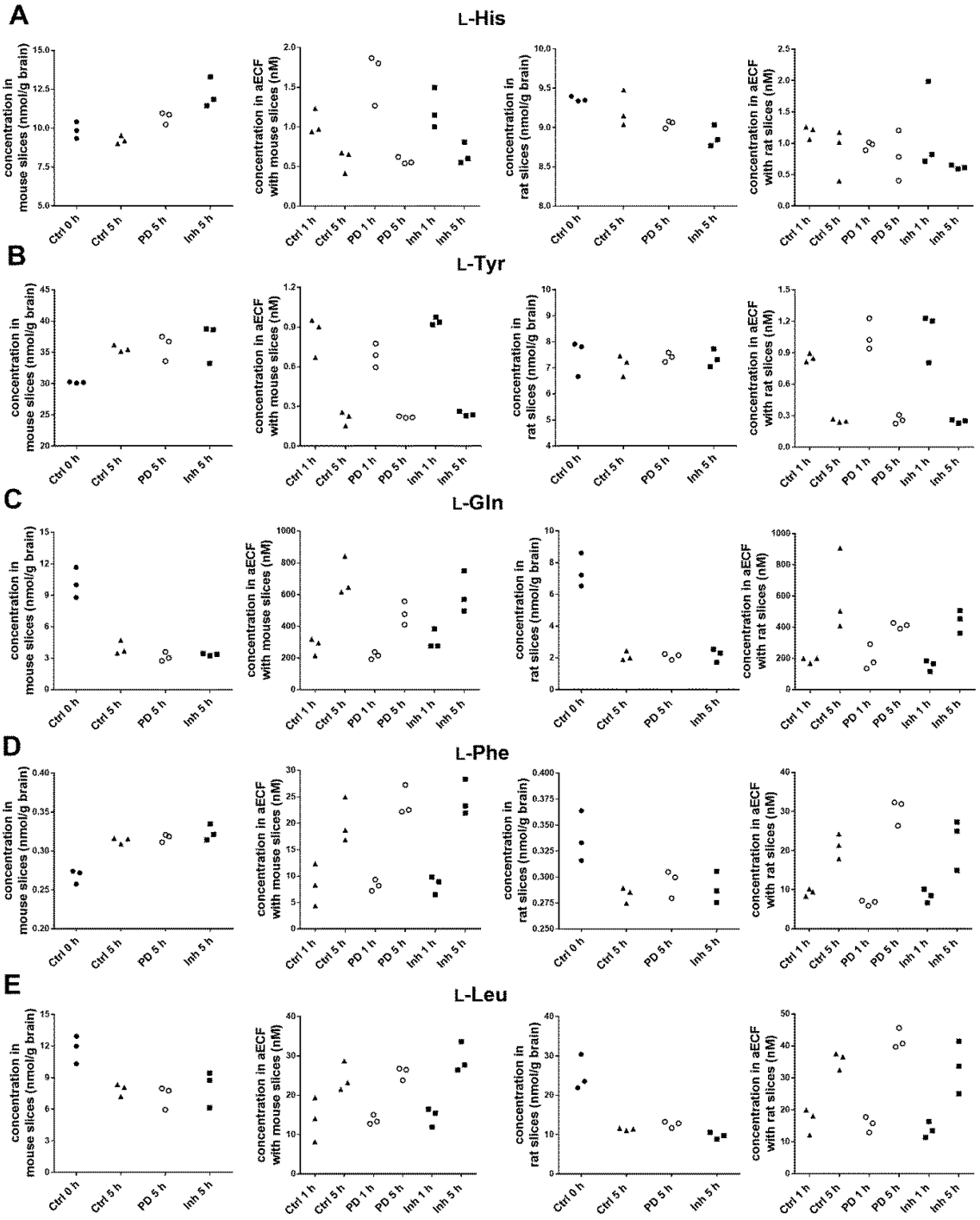

Figure 3. Concentrations of LAT1-utilizing amino acids measured in mouse and rat brain slices as well as aECF: L-His (A), L-Tyr (B), L-Gln (C), L-Phe (D), L-Leu (E). The data is presented for each experiment illustrating the values obtained from the individual animals (n = 3 per group). The concentrations in brain slices were measured at baseline (●, Ctrl 0 h), after 5 h of incubation in aECF (▲, Ctrl 5 h), and after 5 h incubation in aECF containing 200 nM prodrug (○, PD 5 h) or 800 nM LAT1 inhibitor (■, Inh 5 h). The concentrations in aECF were measured after 1 and 5 h of incubation (▲) without compounds and with either the prodrug (○) or the LAT1 inhibitor (■).

brain, respectively, after the 5 h incubation with or without the prodrug or LAT1 inhibitor as compared to control.

There was a change in aECF concentrations of the investigated amino acids observed after the incubation ranging from 1 to 5 h in the mouse and rat brain slices with or without prodrug or LAT1 inhibitor (Figure 3). The aECF concentrations of L-His after 5 h incubation of mouse slices as well as L-Tyr after incubation of either mouse or rat slices decreased by more than 1.5 (L-His) or 3.5 times (L-Tyr). In contrast, the incubation of rat slices with or without prodrug or LAT1 inhibitor did not affect aECF levels of L-His, these ranged from 0.6−1.9 nM. The aECF concentrations of L-Gln, L-Phe, and L-Leu approximately doubled from 1 to 5 h incubation with or without prodrug or LAT1 inhibitor in both species.

Combining NeuroPK Parameters in Mice. The integration of parameters derived from the present intrabrain distribution study, equilibrium dialysis, and the previously reported PK study[15] using the CMA[26] provided important

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Table 2. NeuroPK Parameters Assessed after a Single Dose of 25 μmol/kg i.p. Injection of Either Ketoprofen or the Prodrug in Mice Using the CMA[a]

			prodrug	
	unit	ketoprofen	prodrug	ketoprofen released from prodrug
Experimentally Measured Parameters				
$AUC_{total,brain}$ [b]	nmol·min/g	12.3	4.1	(6.3)
$AUC_{total,plasma}$ [b]	nmol·min/mL	12757	525	521
$K_{p,brain}$		0.0009	0.008	0.012
$f_{u,plasma}$ [b]		0.014 ± 0.001	0.059 ± 0.003	0.014 ± 0.001[c]
Parameters Estimated Using the CMA				
$K_{p,uu,brain}$		0.042	0.006	0.042[c]
$AUC_{u,brainECF}$	nmol·min/g	8.2	0.17	0.32 (4.2)
$AUC_{u,brainICF}$	nmol·min/g	1.61	0.25	0.068 (0.83)
$AUC_{u,plasma}$ [b]	nmol·min/mL	179	31.0	7.3

[a]Data in parentheses is presented for ketoprofen quantified in the brain,[15] which includes both ketoprofen released in plasma and passing into the brain as well as the parent drug, which had been bioconverted in the brain. The total brain concentration of compounds was corrected with respect to the residual blood concentration. [b]Parameters evaluated in the previous study.[15] [c]We assumed the parameter to be equal to the value obtained after ketoprofen dosing.

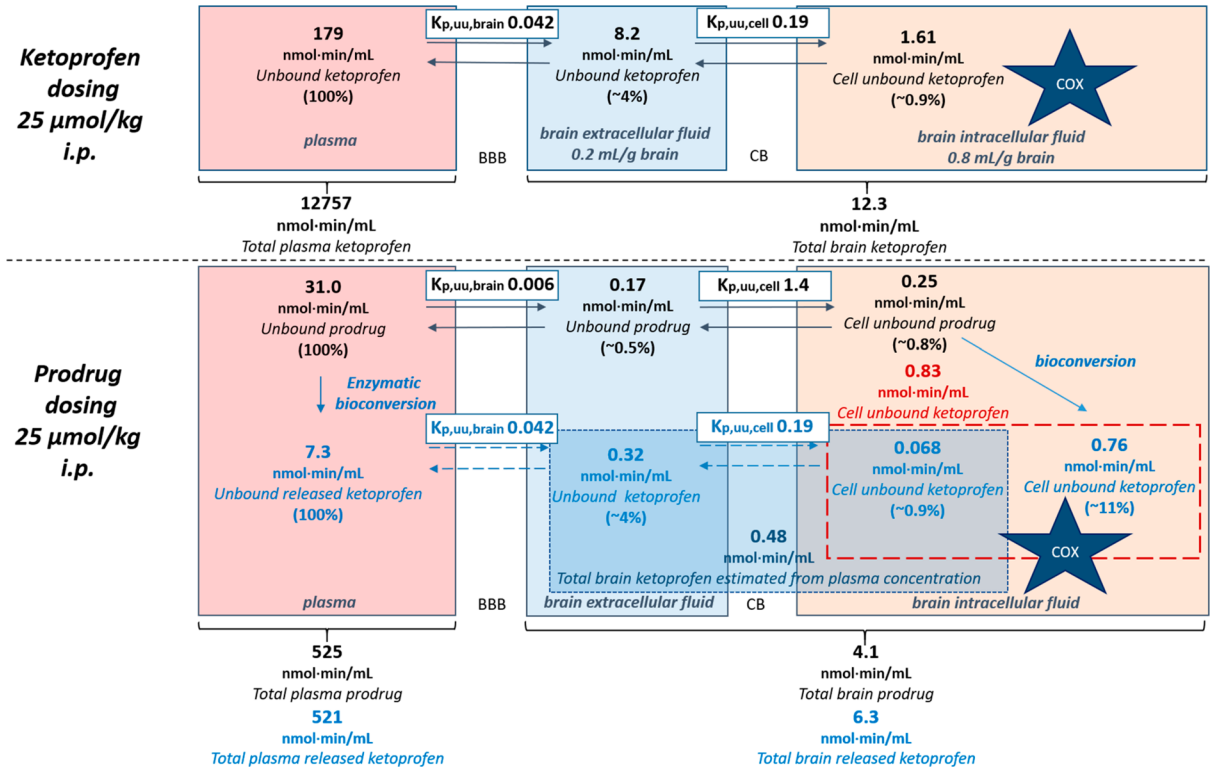

Figure 4. Integrated picture comparing the BBB transport and brain extra- and intracellular distribution of unbound ketoprofen and the ketoprofen prodrug as well as the release of ketoprofen. The cyclooxygenase (COX) target is located in the brain intracellular compartment (indicated by the star). The figure shows the percentage of $AUC_{u,plasma}$ of ketoprofen, prodrug, and parent drug released from prodrug which had been distributed to different brain compartments. The schematic representation was made using the CMA and integration of parameters obtained from the PK study in mice after a single dose of 25 μmol/kg i.p. of ketoprofen or prodrug,[15] brain slice study in mice, and equilibrium dialysis study, where nmol·min/g is assumed to be equal to nmol·min/mL.

information about neuroPK of the prodrug of ketoprofen in mice. Ketoprofen had a more than 8 times lower $K_{p,brain}$ value than the prodrug (Table 2). The $K_{p,brain}$ of ketoprofen was 13 times lower than that of the parent drug released from the prodrug; this is evidence that the ketoprofen detected in the brain after prodrug dosing had been mainly bioconverted in the brain. In contrast, $K_{p,uu,brain}$ of the prodrug was 7 times lower than the corresponding value for ketoprofen. Interestingly, approximately 0.5% and 0.8% of plasma unbound prodrug were distributed to extra- and intracellular compartments, respectively (Figure 4). In contrast, the unbound ketoprofen accounted around 4% and 0.9% of plasma unbound levels in brain ECF and ICF, respectively. Importantly, the parent drug released in the intracellular compartment of the brain parenchyma accounted for approximately 11% of plasma released ketoprofen (Figure 4), demonstrating a significantly greater intracellular distribution between ICF and plasma in comparison to ketoprofen dosing (only 0.9%).

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DISCUSSION

Intrabrain Distribution of Ketoprofen and Prodrug in Mice. The study provided novel mechanistic insights into the neuroPK of the LAT1-utilizing prodrug of ketoprofen focusing on its intrabrain distribution and the release of the parent drug within the brain. The LAT1-utilizing prodrug of ketoprofen, selected from our previous study,[15] achieved a higher extent of intracellular distribution from brain ECF than the parent drug, ketoprofen. Thus, in contrast to ketoprofen ($V_{u,brain}$, 1.5 mL/g brain), the $V_{u,brain}$ of the prodrug (24.3 mL/g brain) was much higher than the volume of brain fluids, i.e., 0.8 mL/g brain. $K_{p,uu,cell}$, which was used to assess the brain cellular barrier transport, was above unity for the prodrug, evidence that the compound was distributed intracellularly, whereas the $K_{p,uu,cell}$ of ketoprofen was below unity, indicative of a predominantly extracellular distribution of the drug.

Importantly, after the incubation with the LAT1 inhibitor, $V_{u,brain}$ and $K_{p,uu,cell}$ of the prodrug decreased by 30%, revealing the involvement of LAT1 in the brain intracellular distribution of the prodrug. The IC_{50} of the LAT1 inhibitor previously measured as the uptake reduction of a known LAT1 substrate [^{14}C]-L-leucine in human breast cancer (MCF-7) cells was 18 μM,[28] which is more than 20 times greater than the concentration used in the brain slice experiments (0.8 μM LAT1 inhibitor). Therefore, in the present study, it was not expected that there would be any remarkable reduction in $V_{u,brain}$ of the prodrug. Interestingly, the $K_{p,uu,cell}$ of the LAT1 inhibitor was 0.045 indicative of a very low intracellular distribution of the compound. This confirms our previous findings that the inhibitor binds reversibly to LAT1 without intracellular accumulation to any meaningful extent.[28] Importantly, both subunits of the transporter, LAT1 and CD98, were quantified in crude membrane fraction of the brain slices. Their protein expression levels were not altered during the 5 h incubation with or without the LAT1 inhibitor (Figure 2). Thus, the reduction in brain intracellular distribution of the prodrug after incubation with the LAT1 inhibitor was not caused by any alteration in the expression of the transporter protein.

Released ketoprofen was not detected during the 5 h incubation with the prodrug, in either mice or rat brain slices. These results are consistent with the previous findings, highlighting the absence of bioconversion of this prodrug in vitro in mouse liver S9 fractions.[15] However, according to the in vivo PK data, the conversion to ketoprofen occurred after single dose injection of prodrug in plasma, liver, and brain in mice.[15] We speculate that the absence of in vitro bioconversion is due to the lack of cofactor(s) of the relevant enzymes in the experimental settings. Additional studies will be needed to identify the metabolizing enzyme(s) and their tissue-specific expression.

NeuroPK Parameters Estimated with the CMA. This is the first time that the brain intracellular distribution of the LAT1-utilizing prodrug has been estimated and compared to that of parent drug using the CMA (Figure 4). Although prodrug had 7 times lower $K_{p,uu,brain}$ than ketoprofen, approximately 1% of plasma levels of both compounds were distributed to the brain parenchymal cells, where the COX enzyme is located. This can be explained by a higher extent of brain intracellular distribution of the prodrug from ECF compared to ketoprofen. The findings are in accordance with the cerebral microdialysis study in rats conducted by Gynther et al.,[22] where the lysine prodrug of ketoprofen had a lower value of $K_{p,uu,brain}$ (0.09) than ketoprofen (0.12), while the overall plasma to brain intracellular delivery efficiency of the prodrug was almost 2-fold greater than ketoprofen.

Importantly, we estimated that the delivery from plasma to the brain intracellular compartment of released ketoprofen was 11 times higher than that after ketoprofen dosing. However, as the CMA used in the evaluation of $AUC_{u,ICF}$ might underestimate this parameter, the difference in the ratio of unbound released ketoprofen in ICF and plasma compared to the parent drug may be even larger. Interestingly, ketoprofen, which had been bioconverted from the prodrug in plasma, contributed only minimally (9%) to the estimated brain intracellular levels of ketoprofen after prodrug dosing. This is convincing evidence that the parent drug had been mostly delivered into the brain parenchyma in the prodrug form.

Previously, we found a five times higher distribution of the released ketoprofen from prodrug between mouse brain and liver compared to ketoprofen dosing.[15] The combination of these findings with 11 times higher brain intracellular distribution of the released ketoprofen as compared to ketoprofen dosing suggests that with a LAT1-utilizing prodrug in mice one can achieve a targeted delivery of ketoprofen to the brain intracellular compartment, where COX is located. This means that it might be possible to administer a sufficiently high dose of the prodrug to achieve effective brain intracellular concentration of the released ketoprofen while not exposing peripheral tissues to toxic ketoprofen levels. This would potentially reduce the drug's systemic adverse effects. In case of proven efficacy of ketoprofen in neurodegenerative diseases such as AD, the prodrug strategy can be promising for improving brain delivery of this drug in patients.

Thus, as exemplified by ketoprofen prodrug in the present study, the LAT1-utilizing prodrug approach can enable targeted brain delivery of drugs, in particular into the brain parenchymal cells with reduced peripheral exposure to the released parent drug. Although these findings should be confirmed in a bigger set of prodrugs of various CNS drugs, the study highlights the importance of the approach application for the drugs with the brain intracellular rather than extracellular targets.

Effect of Prodrug on LAT1 Protein Expression and Function. As transporters play a pivotal physiological role in the delivery of endogenous compounds, the development of influx-transporter utilizing prodrugs has to be based on an understanding of impact of the prodrug on the transporter function and protein expression. Here, we report for the first time that the LAT1-utilizing prodrug of ketoprofen had no effect on the protein expression of light (LAT1) and heavy chain (CD98) subunit of the transporter in crude membranes of mouse and rat brain slices after the 5 h incubation (Figure 2). In addition, during the incubation, there was the exchange of four essential amino acids (L-Phe, L-Leu, L-His, L-Tyr) and a nonessential L-Gln between brain slices and aECF providing evidence that LAT1 was functional in both species. Thus, L-His and L-Tyr concentrations in aECF decreased throughout the incubation from 1 to 5 h highlighting their participation in the antiport of other amino acids. When the latter amino acids reach equilibrium, L-His and L-Tyr accumulated in brain slices showing the same levels after the 5 h incubation. In contrast, L-Phe, L-Leu, and L-Gln concentrations in aECF doubled during the 5 h incubation in mice and rats. The brain slice concentrations of L-Gln and L-Leu decreased, while the levels

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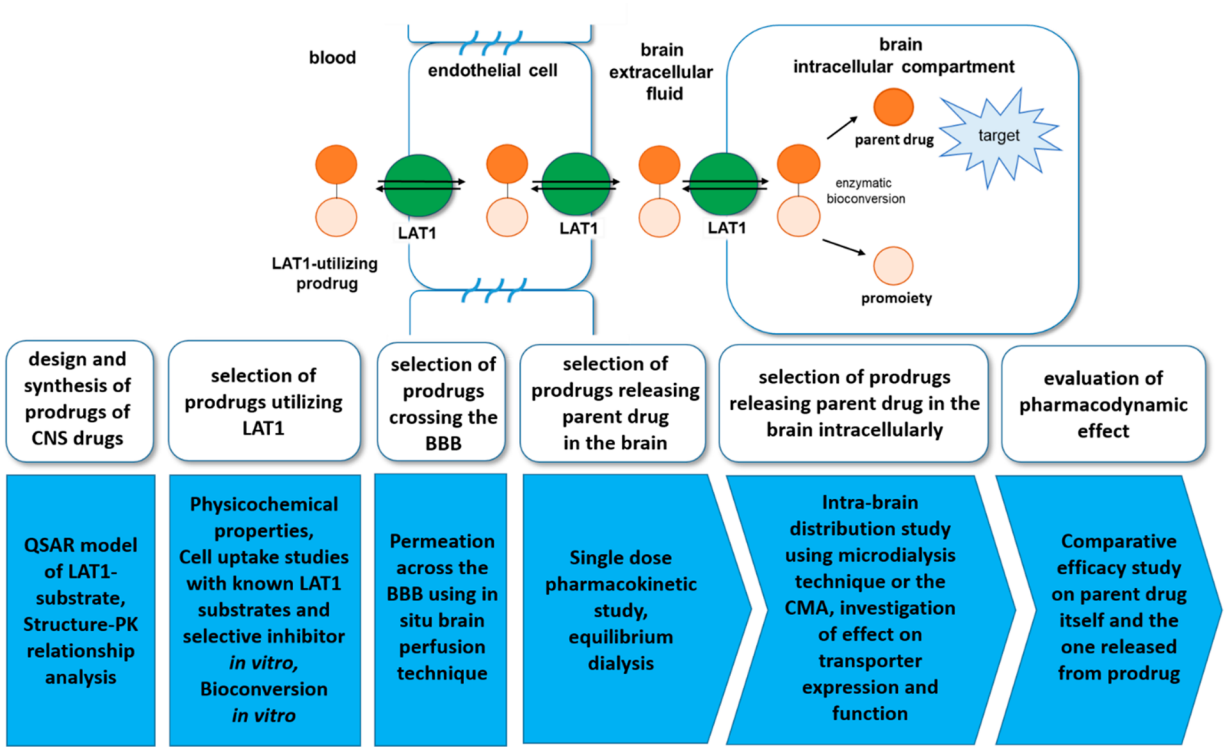

Figure 5. Proposed approach to preclinical development of influx transporter-utilizing prodrugs exemplified by LAT1 for the brain delivery of drugs with intracellular targets.

of L-Phe in brain slices remained the same, demonstrating the efflux of these amino acids to the aECF. The results of our study are in accordance with the current knowledge on the function of the LAT1, i.e., this transporter mediates the inward flux of His, Leu, Phe, Gln, Tyr, and other amino acids in antiport with His, Gln, and Tyr with 1:1 stoichiometry (Figure 1).[40,41] One important result emerging from this study is that the distribution of the prodrug into the brain slices did not alter the exchange of the investigated amino acids in either species.

Optimal Strategy for the Preclinical Development of Influx Transporter-Utilizing Prodrugs. Previously, we proposed a screening strategy for the development of influx-transporter utilizing prodrugs such as those exploiting LAT1; this involved the selection of prodrugs based on their utilization of the transporter for cellular uptake in vitro, BBB permeation, and preclinical PK.[15] In the current study, we refined the strategy for development of prodrugs and now propose some additional steps including investigating the intrabrain distribution as well as determining the impact of prodrugs on the physiological state of the transporter (Figure 5). Here, we will discuss the interpretation of the important neuroPK parameters, which should be considered while developing influx-transporter utilizing prodrugs for the brain delivery of drugs with intracellular targets.

The single dose PK study is essential for the investigation of the BBB transport extent of unbound prodrugs utilizing LAT1 and, more importantly, for the evaluation of the release of parent drug inside the brain. The extent of BBB transport of the unbound prodrug can be estimated by measuring $K_{p,brain}$ or calculating $K_{p,uu,brain}$.[42] The latter parameter can be assessed by direct measurement of the unbound prodrug in plasma and brain ECF either using cerebral microdialysis techniques or estimated with the CMA.[26] It is important to bear in mind that $K_{p,brain}$ is affected by the nonspecific binding of the compound to different components within the brain tissue and blood, while $K_{p,uu,brain}$ characterizes the net passive and active drug transport and equilibration across the BBB. Thus, the 8-fold higher $K_{p,brain}$ of the prodrug as compared to ketoprofen revealed in this study could be misleading and interpreted as higher BBB transport of the prodrug. In contrast, a true measure of the BBB transport, $K_{p,uu,brain}$, revealed that the prodrug had a 7 times lower extent of the BBB transport compared to ketoprofen. This variation is mainly due to differences in brain binding and intrabrain distribution.

Although $K_{p,uu,brain}$ is a parameter widely used for CNS drug candidate screening, basing the selection of prodrugs merely on their $K_{p,uu,brain}$ values can be misleading. One should remember that despite a low $K_{p,uu,brain}$, prodrugs might have higher extent of brain cellular barrier transport ($K_{p,uu,cell}$) compared to the parent drug, which governs the brain intracellular distribution of prodrugs followed by their potential bioconversion in the brain intracellular compartment. These latter properties are in fact desirable for the development of LAT1-utilizing prodrugs. Therefore, in a prodrug approach, the focus should be placed on assessing both the $K_{p,uu,brain}$ value of a prodrug as well as the intrabrain distribution of the prodrug and the release of parent drug within the brain. Thus, during the selection of prodrugs, it is recommended that compounds with values of $K_{p,uu,cell}$ greater than unity due to the confirmed transporter related uptake should be prioritized. This can be determined in vitro by using a combination of brain slice and brain homogenate studies, even before embarking on in vivo studies.

Another key issue in the development of the transporter-utilizing prodrugs is understanding of interspecies differences in the tissue-specific protein expression of the transporter and enzyme(s) responsible for the bioconversion of the prodrug.

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For example, differences in the transporter and enzyme expression may eventually result in interspecies variations in brain delivery of prodrug and the release of parent drug, respectively. While limited information about the prodrug metabolizing enzymes in the brain is available, the LAT1 protein expression has been quantified in plasma membranes of cortex microvessels isolated from humans (0.43 ± 0.09 fmol/μg protein), mice (2.19 ± 0.21 fmol/μg protein), and rats (3.00 ± 0.62 fmol/μg protein).[9,10] In addition, LAT1 protein expression has been detected in human and mouse astrocytes, and its mRNA levels were detected in mouse and rat neurons.[10,12,37] In the present study, the protein expression of LAT1 was the same in crude membrane of mouse and rat brain slices. The minor difference in intrabrain distribution of the prodrug with slightly higher $V_{u,brain}$ in rats (31.4 mL/g brain, respectively) than in mice (24.3 mL/g brain, respectively) is in accordance with the values obtained for the protein expression levels of LAT1 in crude membranes from these two species. Moreover, the incubation of mouse and rat brain slices with the LAT1 inhibitor resulted in a similar reduction of the $V_{u,brain}$ and $K_{p,uu,cell}$. The changes in amino acid concentrations were similar in all experimental groups in both species. Overall, these results indicate that the intrabrain distribution of the LAT1-utilizing prodrug was similar in mice and rats.

Importantly, in the present study, the estimation of the concentrations of prodrug and released parent drug in the brain intra- and extracellular compartments has been done assuming steady-state. However, as concentration−time profiles at the target site drive the interaction with the target(s), the dynamic information about pharmacokinetics of prodrugs in different body compartments is essential, and it has to be studied in the future in order to understand and predict pharmacokinetic−pharmacodynamic relationships.

Finally, the valid application of the proposed approach will require an assessment of transporter function and expression not only in physiological but also under pathological conditions and the subsequent investigation of the pharmacodynamic effect of the prodrugs (which *per se* are inactive) compared to parent drug in a relevant (animal) disease model. In the present study, inhibition of LAT1 was found to modify the intrabrain distribution of the prodrug by decreasing its brain intracellular delivery. This suggests that up- or down-regulation of the transporter expression in pathological conditions could affect the brain delivery of transporter-utilizing prodrugs. Recently, Gynther et al. showed that LAT1 function in both mouse BBB and in mouse primary astrocytes remain unaltered after LPS-induced inflammation or in the presence of gene mutations in either amyloid precursor protein (*APP*) or presenilin (*PSEN1*) mimicking AD.[37] If this holds true in humans, then one could speculate that the transporter would be capable of brain delivering therapeutic compounds including ketoprofen used in the present study, even in pathological conditions such as AD.

In conclusion, we have determined mechanistic and quantitative information about the intrabrain distribution of a LAT1-utilizing prodrug of ketoprofen used as a tool to probe brain distribution of influx transporter-utilizing prodrugs, by using a brain slice method in mice and rats. This is the first time that the brain slice method has been exploited for investigating the impact of influx transporter on intrabrain distribution of prodrug. The LAT1-mediated prodrug of ketoprofen was delivered to the brain intracellular compartment where its target, the COX enzyme, is located. Importantly, the distribution of the LAT1-utilizing prodrug did not affect the expression or function of the transporter. Finally, we propose additional steps to the strategy for the effective development of prodrugs utilizing influx transporters exemplified by LAT1 for targeted delivery of drugs into the brain. These include investigating the intrabrain distribution of prodrugs and released parent drug, as well as evaluating the influence of the prodrugs on the transporter protein expression and function. Overall, this study illustrated that in addition to a reduction in the peripheral delivery of the parent drug, the LAT1-utilizing prodrug approach can be a promising way of delivering drugs into the intracellular compartment of brain parenchymal cells.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.9b00502.

Chemical structures of ketoprofen, prodrug, and LAT1 inhibitor (Figure S1); the uptake dynamics of ketoprofen, the prodrug, and the LAT1 inhibitor in aECF during the experiments in brain slices (Figure S2); probe peptide amino acid sequences and MRM transitions for the LC−MS/MS analysis of target proteins (Table S1); absolute protein expression levels of LAT1, CD98, GLUT1, and Na^+/K^+-ATPase measured in the crude membrane fractions of brain slices (Table S2) (PDF)

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Notes

The authors declare no competing financial interest.

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