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FULL PAPER

The effects of prodrug size and a carbonyl linker on L-type amino acid transporter 1-targeted cellular and brain uptake

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Abstract: The L-type amino acid transporter 1 (LAT1, SLC7A5) imports dietary amino acids and amino acid drugs (*e.g.* L-DOPA) into the brain, and it plays a role in cancer metabolism. Though there have been numerous reports of LAT1-targeted amino acid-drug conjugates (prodrugs), identifying the structural determinants to enhance substrate activity has been challenging. In this work, we investigated the position and orientation of a carbonyl group in linking hydrophobic moieties including the anti-inflammatory drug ketoprofen to L-tyrosine and L-phenylalanine. We found that esters of *meta*-carboxyl L-phenylalanine had better LAT1 transport rates than the corresponding acylated L-tyrosine analogs. However, as the size of the hydrophobic moiety increased, we observed a decrease in LAT1 transport rate with a concomitant increase in potency of inhibition. Our results have important implications for designing amino acid prodrugs that target LAT1 at the blood-brain barrier (BBB) or on cancer cells.

Introduction

The L-type amino acid transporter 1 (LAT1, SLC7A5) is a sodium-independent heterodimeric transmembrane protein highly expressed in both the blood-brain barrier[1] (BBB) and in numerous cancer types.[2] LAT1 transports neutral amino acids (*e.g.*, Phe, Leu, Met) and L-histidine as well as amino acid-containing drugs such as gabapentin,[3] melphalan,[4] and L-DOPA,[5] which are used for treating brain disorders. Additionally, drugs conjugated to phenylalanine (prodrugs) may also be LAT1 substrates.[6]

A major challenge of designing LAT1-targeted amino acid prodrugs is that optimization of ligand-transporter interactions can lead to inhibition rather than transport into the cell.[7] We have shown that the structural differences between an inhibitor and substrate are subtle.[8] For example, we[7a] and others[9] found that substitution of phenylalanine's aromatic ring at the meta position generally resulted in improved uptake rate and/or binding potency relative to the ortho and para positions. However, meta substitution by larger, hydrophobic groups (*e.g.*, phenyl or benzyl) resulted in good LAT1 inhibition (IC_{50} 5-10 μM) but not transport.[7a] Moreover, amino acids containing 4-5 aromatic rings have been reported as highly potent LAT1 inhibitors rather than substrates.[10]

We previously showed that LAT1 substrate SAR was surprisingly tolerant of various functional groups at phenylalanine's meta position.[8] We also found that there was not a direct correlation between the polarity of the meta substituent and a ligand's affinity. For instance, both *tert*-butyl- and hydroxyethyl-substituted esters had comparable IC_{50} values and transport rates. However, when a *tert*-butyl group was directly attached at the meta position without an ester linkage, a substantial decrease in transport rate was observed.[8] Additionally, other carbonyl-containing functional groups (*e.g.*,

amide, ketone, and carboxylic acid) resulted in substrate activity. Given the carbonyl linker's positive effect on substrate activity for relatively small substituents, we hypothesized that larger moieties might also benefit from its presence. Furthermore, since carbonyl groups are a commonly used structural motif for attaching drugs to amino acids in LAT1-targeted drug delivery,[6a,9b,11] it would be advantageous to optimize their position and orientation within a prodrug to increase its substrate activity. That combined with the size and shape of the LAT1 binding site from recently solved structures[12] and analysis of docked ligands[8] led to the idea of studying the effects of changing the following variables for a carbonyl linker:

1. position in relation to an aromatic "drug" (Fig. 1a, "drug" = phenyl) compared with phenylalanine's aromatic ring (Fig. 1b) and the amino acid moiety;
2. distance of the carbonyl-substituted aromatic "drug" from the amino acid moiety (Fig. 1a: variable chain length, n);
3. mode of attachment (Fig. 1b vs. 1c) for larger, hydrophobic moieties ("drug" = phenyl or ketoprofen) compared with a methyl group.

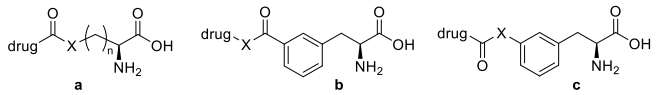


Figure 1. Some general strategies for attaching drugs to amino acids involving carbonyl groups (X = NH or O). (a) Carboxyl-containing drug conjugated to lysine (X = NH, n = 4), serine (X = O, n = 1) or their homologs (variable n).[13] (b) Alcohol- or amine-containing drug conjugated with *meta*-carboxyl phenylalanine.[10b,11b,14] (c) Carboxyl-containing drug conjugated with *meta*-tyrosine (X = O) or its anilino analog (X = NH).[9b,13b,15]

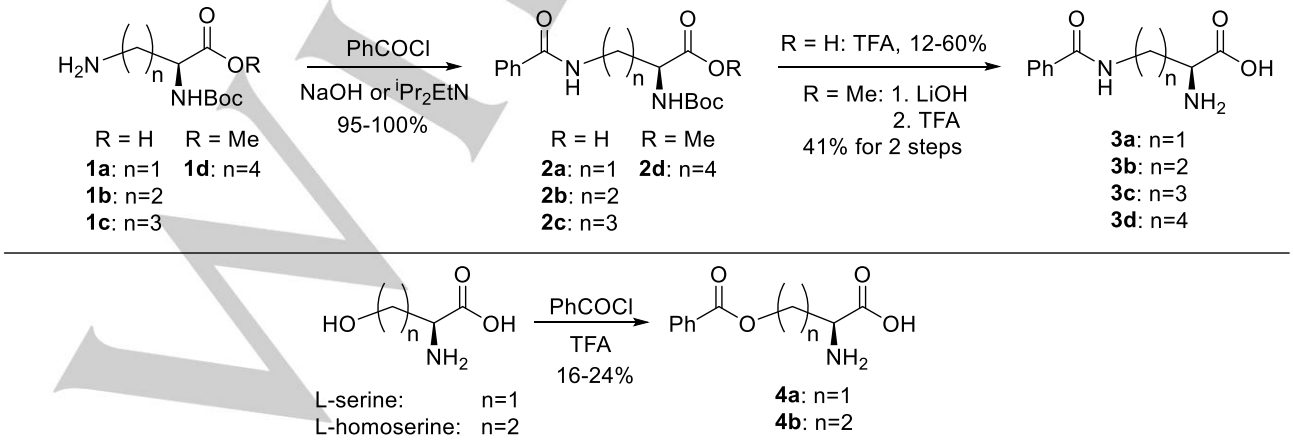
To address variables 1 and 2, we prepared a series of benzoyl esters and amides derived from serine and lysine homologs (Scheme 1). It had been previously shown that an amide of ketoprofen and lysine (analogous to compound **3d**) resulted in 79% inhibition of the uptake of [^{14}C]-L-leucine in a rat brain perfusion experiment and was able to cross the BBB.[16] However, it was not directly demonstrated whether this compound was a LAT1 substrate or inhibitor or whether it might be gaining access to the brain *via* a different transporter. Moreover, there has not been a systematic study of the effect of chain length for substituted serine and lysine homologs on LAT1 activity. It should also be noted that "reverse amide" analogs of **3a** and **3b** (*i.e.* derived from aspartic and glutamic acid) have been described, and they were shown to be relatively weak binders (0% and 57% inhibition of [^{14}C]-L-leucine uptake in rat brain perfusion experiments, respectively).[17] Thus, we did not pursue this type of carbonyl linkage in the current study.

To address variable 3, we prepared hydrophobic esters, containing phenyl or ketoprofen moieties, employing two different modes of attachment (Schemes 2-4). We selected ketoprofen, a nonsteroidal anti-inflammatory drug, because the LAT1 activity of ketoprofen-tyrosine conjugates (*i.e.*, **21** and **22**) had previously been described.[6a,15] Moreover, the BBB permeability of prodrug **21** had been correlated with LAT1 uptake.[6a] Our models have predicted the presence of polar side chains near the binding site (*e.g.*, Ser143, Ser338, Asn404) that are capable of hydrogen bonding to a carbonyl substituent potentially leading to enhanced potency.[8] Thus, from the results of our previous SAR study[8] (for example, **10** vs. **11**), we hypothesized that reversing the orientation of the ester linkage as in **23** by employing a ketoprofen derivative (alcohol **17**) would lead to enhanced LAT1 potency while maintaining uptake rate, resulting in greater BBB permeability relative to **22**. Likewise, we prepared and tested phenoxycarbonyl analog **13** and benzoyloxy-substituted phenylalanine derivatives **14** and **15** to determine the effect of the ester's mode of attachment for an intermediate-sized substituent between the larger ketoprofen and a methyl group (*i.e.*, **10** and **11**).

Results and Discussion

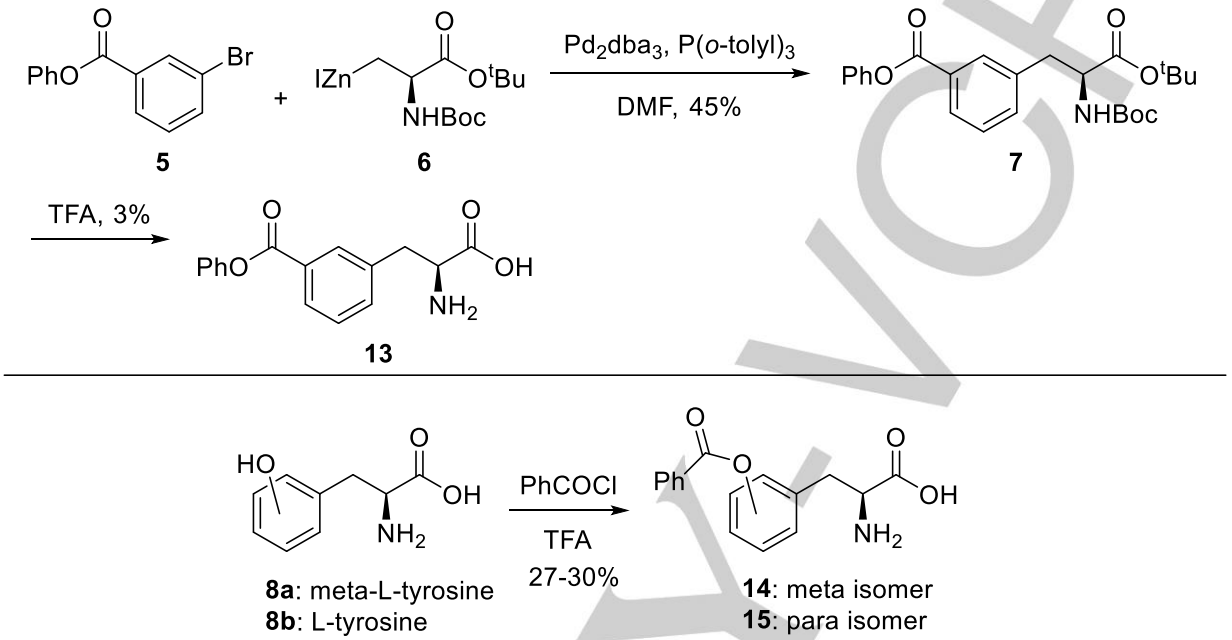
Synthesis

The syntheses of benzoyl amide and ester homologs of Table 1, **3a**-**d** and **4a**-**b**, respectively, were conveniently performed according to Scheme 1. Benzoylation of commercially available BOC-protected lysine homologs **1a**-**c** (R = H) provided amides **2a**-**c**. Lysine derivative **2d** (R = Me) was prepared from methyl ester **1d** due to the convenience of availability at the time we initiated the synthesis. Deprotection of **2a**-**d** gave desired lysine homologs **3a**-**d**. Esters **4a**-**b** were easily prepared in one step by reaction of L-serine or L-homoserine with benzoyl chloride in neat TFA to avoid reaction at the alpha amino group.



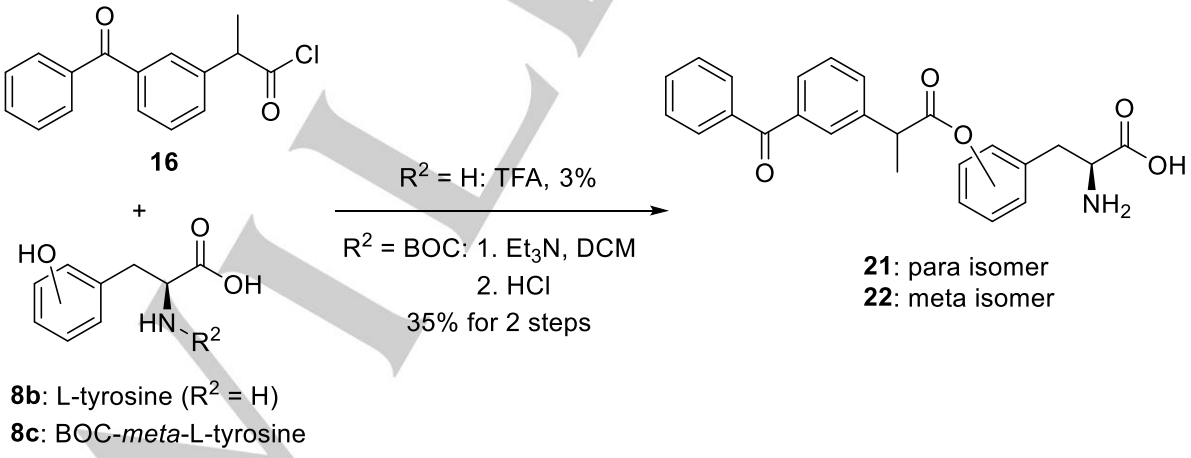
Scheme 1. Synthesis of benzoyl amides **3a**-**d** and esters **4a**-**b**.

Phenoxycarbonyl- and benzoyloxy-substituted phenylalanine derivatives of Table 2 (**13-15**) were synthesized according to Scheme 2. The syntheses of all other phenylalanine derivatives of Table 2 have previously been described.[7a,8] Negishi coupling between aryl bromide **5**, synthesized by esterification of commercially available 3-bromobenzoic acid with phenol, and organozinc **6**[8] gave protected phenylalanine derivative **7**. BOC and *tert*-butyl groups were conveniently removed in one step using TFA to give desired phenoxycarbonyl analog **13**. Though benzoyloxy analog **15** was previously synthesized in multiple steps using protecting groups,[17] we found that in a manner analogous to **4a-b** both benzoyloxy analogs **14** and **15** could be simply prepared by benzoylation of *meta*-L-tyrosine and L-tyrosine in neat TFA, respectively.



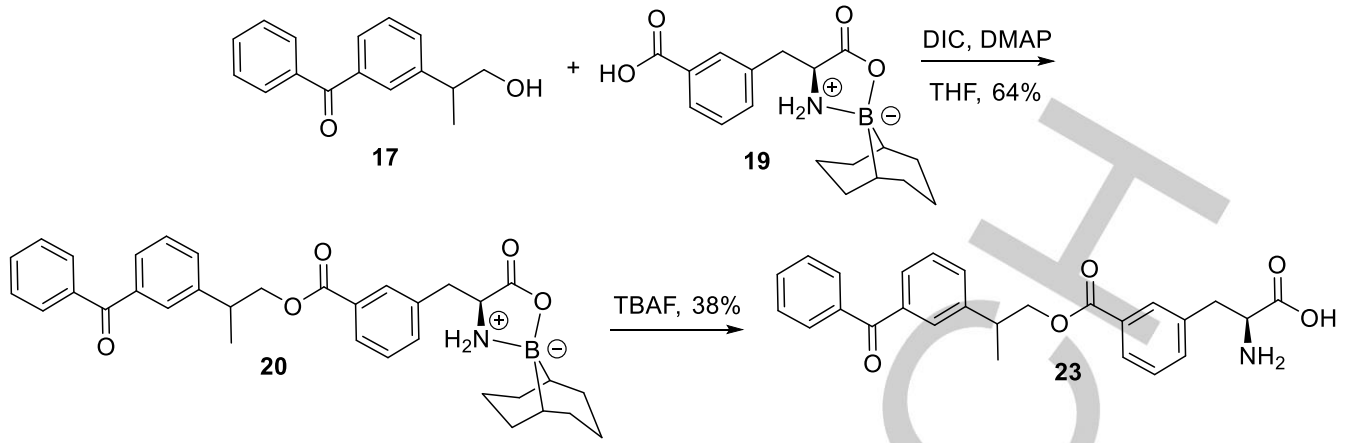
Scheme 2. Synthesis of phenoxycarbonyl analog **13** and benzoyloxy-substituted phenylalanine derivatives **14** and **15**.

The para-substituted ketoprofen-tyrosine conjugate **21** was synthesized by acylation of L-tyrosine in TFA solvent as previously described (Scheme 3).[6a] However, in our hands, only a 3% yield of **21** was attained after preparative HPLC purification. We found that a significantly higher yield (35%) could be achieved for the synthesis of meta isomer **22** by acylation of BOC-protected meta-L-tyrosine **8c**, followed by deprotection using HCl in dioxane.



Scheme 3. Synthesis of para- and meta-substituted ketoprofen-tyrosine conjugates **21** and **22**.

Compound **23**, a "reverse ester" analog of the meta-substituted ketoprofen-tyrosine conjugate **22**, was prepared according to Scheme 4. Alcohol **17** was obtained using a borane reduction of ketoprofen.[18] DIC-mediated coupling of alcohol **17** and 9-BBN-protected **19**, derived from *meta*-carboxyl phenylalanine **18** (not depicted),[11b] gave ester **20**, which was subsequently deprotected using TBAF[19] to provide desired analog **23**.



Scheme 4. Synthesis of ketoprofen-derived "reverse ester" analog **23**.

SAR from HEK-hLAT1 Cell Assays

To determine LAT1 activity of our compounds, both *cis*-inhibition and *trans*-stimulation assays were performed using HEK cells that overexpress human LAT1, as previously described.[8,20] Both positive and negative controls (*i.e.*, phenylalanine and arginine, respectively) were used in cell assays. A *cis*-inhibition assay allows for evaluation of LAT1 potency. The %inhibition data were determined by measuring the uptake of a radiolabeled substrate, [^{3}H]-gabapentin, in the presence of test compounds. IC_{50} values were determined for selected compounds. Though a *cis*-inhibition assay is vital for determining ligand potency, it is insufficient for determining whether a compound is a LAT1 substrate. A *trans*-stimulation assay was performed for determining whether a compound is a LAT1 substrate. This assay takes advantage of LAT1's alternating-access mechanism.[21] In brief, HEK-hLAT1 cells were pre-loaded with [^{3}H]-gabapentin and then incubated with test compounds. Because there is a 1:1 stoichiometry for exchange, the measured efflux rate of [^{3}H]-gabapentin is directly related to the rate of substrate uptake.[3,22] For ease of comparison, the compounds' transport rates were normalized to the value for L-phenylalanine (2.7 ± 0.3 fmol/min), which was set to 100%. For a compound to be considered a substrate, its transport rate must be greater than the background efflux rate of [^{3}H]-gabapentin, as determined in the presence of negative control. The SAR data from these two assays serve as a predictor of a compound's LAT1 activity *in vivo*.

Benzoylated serine **4a** and homoserine analog **4b** (Table 1) exhibited greater LAT1 transport rates than amides **3a** and **3b** with the same chain length, as evidenced by larger %efflux of [^{3}H]-gabapentin in our *trans*-stimulation assay. However, both amides (**3a-b**) and esters (**4a-b**) with shorter chain length (n = 1, 2) had poor potency (%inhibition of LAT1 at 200 μM: 4.6–16%). As the chain length was increased to 3 carbon atoms for amide **3c**, there was an increase in binding potency. This agrees with its docking pose (Fig. 2A), which suggests an additional pi-pi interaction between its aromatic sidechain and Y259. Additionally, **3c** had a better docking score than all other compounds of Table 1 (supporting information, Table S1). In contrast, an interaction with Y259 is not predicted for **3a** or **3b** (Fig. 2B), both of which are less potent than **3c**. While **3d**, containing a chain of 4 carbon atoms, is also predicted to make a pi-pi interaction with Y259, its diminished potency may be due to an entropic penalty resulting from the additional carbon atom. Transport rates for **3c** and **3d** were comparable to negative controls L-lysine and L-serine indicating that they are more likely to be inhibitors than substrates.

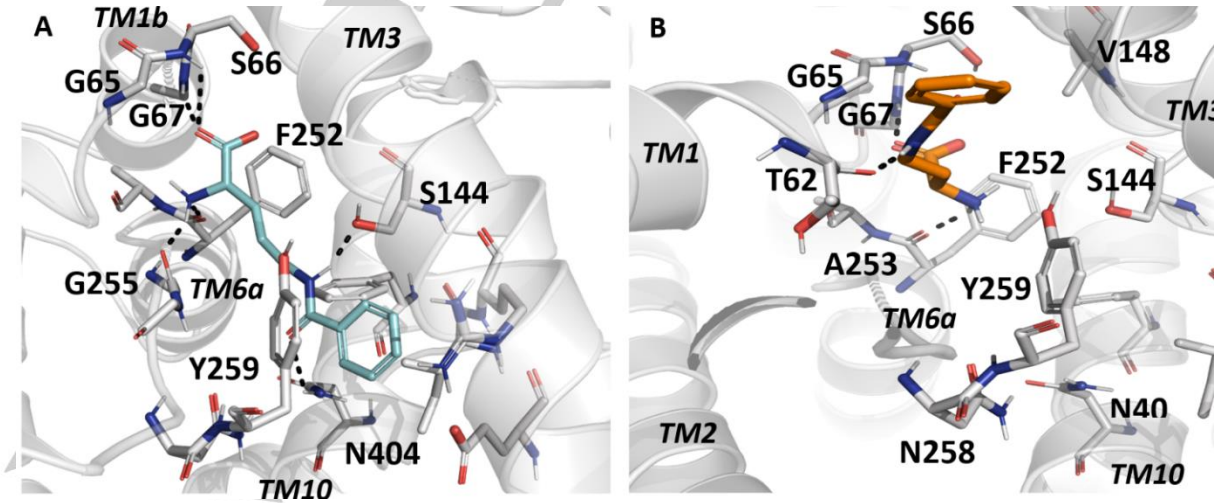
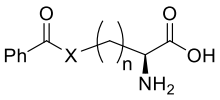


Figure 2. Docking poses of compounds **3c** (A) and **3b** (B). Residues predicted to make hydrogen bonds (black dashes) are labeled. Oxygen, nitrogen, and carbon atoms are represented in red, blue, and white, respectively.

The only amide of Table 1 that was a LAT1 substrate was **3a**, which possessed the shortest chain length (n = 1). Although esters **4a**-**b** were substrates, they demonstrated significantly lower potency (11-16% vs. 85% inhibition) and transport rates (56-59% vs. 100% efflux) than positive control L-phenylalanine. Due to the lack of commercial availability of synthetic precursors and potentially difficult syntheses, longer chain homologs of serine (*i.e.* X = O, n = 3 and 4) were not pursued.

Taken together with our previous SAR study[8] and the results of Table 2, it is apparent that positioning the carbonyl linker between the aromatic ring and amino acid backbone as in serine and lysine homologs of Table 1 results in decreased potency relative to carbonyl at the meta position (*e.g.*, compounds **10** and **13**). However, given that **3a** and **4a-b** are substrates, conjugation of drugs with lysine and serine homologs may still be useful for designing LAT1-targeted prodrugs. Also, it would probably be more convenient to synthesize amino acid prodrugs from lysine or serine and their homologs according to Scheme 1 than from meta-substituted phenylalanine derivatives (Schemes 2-4)**.**

Table 1. Relative exchange efflux rate and uptake inhibition of [^{3}H]-gabapentin in HEK-hLAT1 cells for benzoylated lysine (**3a-3d**) and serine (**4a-b**) homologs with variable chain length.

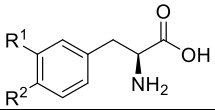


Compound[a]	X	n	%L-Phe Efflux[b]	%Inhibition[c]
L-Phe	-	1	100	85
L-Lys	-	4	33	-2.1
L-Ser	-	1	35	-1.0
3a	N	1	47	4.6
3b	N	2	37	8.6
3c	N	3	34	54
3d	N	4	26	38
4a	O	1	59	16
4b	O	2	56	11

[a] Cell assay data was obtained at least in triplicate (wells). Amino acids were purchased from commercial vendors or synthesized as depicted in Scheme 1. All compounds above are single enantiomers of L configuration. [b] Compounds were tested at 200 μM for their ability to cause efflux (fmol/min) of [^{3}H]-gabapentin from pre-loaded HEK-hLAT1 cells. Efflux of [^{3}H]-gabapentin was calculated at 3 min after adding test compound. %Efflux was normalized relative to L-Phe, which had an efflux rate of 2.7 ± 0.3 fmol/min, from an average of seven experiments. [c] Compounds were tested at 200 μM for their ability to inhibit uptake of [^{3}H]-gabapentin into HEK-hLAT1 cells. Data is presented as % inhibition relative to background signal in the absence of a test compound.

Consistent with our previous SAR study (*e.g.* **10** vs. **11**),[8] having the carbonyl group directly attached to phenylalanine's aromatic ring in phenoxycarbonyl analog **13** resulted in a greater transport rate than benzoylated *meta*-tyrosine **14**. The latter is likely a LAT1 inhibitor rather than a substrate due to its poor transport rate. As with methyl ester **12**, substitution at the para position in **15** led to a decrease in potency, which is consistent with previous reports that the meta position is preferred for enhancing LAT1 affinity.[7a,9b] Surprisingly, the bulkier phenyl ester **13** had both a lower transport rate and potency (higher IC_{50}) relative to its methyl ester counterpart **10**, whereas **14** showed significantly greater potency compared with its methyl analog **11**. Nonetheless, the substrate activity of phenyl ester **13** supports our hypothesis that employing an ester linkage in which the carbonyl is directly attached to the aromatic ring of phenylalanine allows for transport of hydrophobic substituents (at least as large as a phenyl group) that otherwise might lead to LAT1 inhibition (*e.g.* **9** vs. **13**). Though our models have predicted that the ester carbonyl of **13** may be able to form hydrogen bonds with residues Ser 143, Ser 348, and Asn 404 in the binding site,[8] we are unable to rationalize the dramatic differences in transport rate and potency depending on the ester's orientation (*e.g.* **13** vs. **14**). Additionally, while **13** and **14** have a similar pose to those of **3c** and **3d**, they are predicted to make additional hydrophobic interactions in the binding site (supporting information, Figure S1). Interestingly, **14** has a slightly better docking score than that of **13** (supporting information, Table S1), in agreement with the experimental results (Table 2).

Table 2. Relative exchange efflux rate, uptake inhibition of [^{3}H]-gabapentin and IC_{50} values in HEK-hLAT1 cells for meta- and para-substituted phenylalanine derivatives **9**-**15** compared with positive and negative controls, L-phenylalanine and L-arginine, respectively.



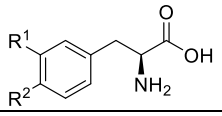
Compound[a]	R^1	R^2	%L-Phe Efflux[b]	%Inhibition[c]	IC_{50} (μM)[d]
L-Phe	H	H	100	85	69 ± 29
L-Arg	-	-	28	49	-
9	$PhCH_2$	H	29	98	7.3 ± 3
10	MeO_2C	H	89	81	36 ± 23
11	$MeCO_2$	H	37	54	-
12	H	MeO_2C	67	37	260 ± 90
13	PhO_2C	H	52	85	70 ± 24
14	$PhCO_2$	H	30	100	11 ± 3
15	H	$PhCO_2$	36	49	-

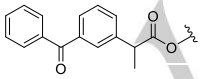
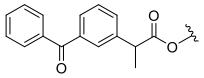
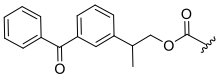
[a] Cell assay data was obtained at least in triplicate (wells). L-Phenylalanine and L-arginine were purchased from commercial vendors. Compounds **13-15** were synthesized as depicted in Scheme 2. We have previously published the synthesis and LAT1 activity of all other amino acids of Table 2.[7a,8] [b,c] Compounds were tested in *trans*-stimulation and *cis*-inhibition cell assays as described for Table 1. [d] For IC_{50} determinations, varying concentrations of each compound were added, from 0.1 μM to 500 μM. IC_{50} and standard deviation of each compound were calculated by Graphpad Prism version 5.0. %[^{3}H]-Gabapentin uptake at each concentration was normalized relative to %inhibition by BCH[23] at 2 mM, which was set to 100% inhibition.

Given the differences in activity between regioisomers **13** and **14**, we were curious as to whether these results would transfer to a larger substituent than phenyl. As indicated above, we selected ketoprofen and a ketoprofen derivative **17** as

substituents, because ketoprofen prodrugs **21** and **22** had previously been reported as LAT1 substrates.[6a,15] We hypothesized that reversing the orientation of the ester linker (*i.e.* **22** vs. **23**) would improve LAT1 transport rate leading to greater brain uptake. Despite reports that both **21** and **22** are transported by LAT1,[6a,15] based on the results of our *trans*-stimulation assay in HEK-hLAT1 cells (Table 3), only compound **21** had a significantly greater %efflux rate than negative control L-arginine. Additionally, **21** and **22** were >50-fold more potent than L-phenylalanine, which is consistent with previous observations that potency is indirectly correlated with LAT1 substrate activity.[7] Interestingly, reversing the orientation of the ester in **23** resulted in an even greater increase in potency (IC_{50} = 0.31 ± 0.1 μM) but did not provide the desired substrate activity that had been observed with phenyl ester **13**. This result indicates that like **21** and **22**, "reverse ester" **23** is most likely an inhibitor and not a substrate, and it is among the more potent LAT1 inhibitors reported in the literature.[10,24]

Table 3. Relative exchange efflux rate, uptake inhibition of [^{3}H]-gabapentin and IC_{50} values in HEK-hLAT1 cells for meta- and para-substituted ketoprofen-tyrosine conjugates **21**, **22**, and "reverse ester" analog **23**.



Compound[a]	R^1	R^2	%L-Phe Efflux[b]	%Inhibition[c]	IC_{50} (μM)[d]
L-Phe	H	H	100	85	69 ± 29
L-Arg	-	-	28	49	-
21	H		45	108	1.2 ± 0.2
22		H	23	107	1.2 ± 0.2
23		H	32	108	0.31 ± 0.1

[a] L-Phenylalanine and L-arginine were purchased from commercial vendors. Compounds **21** and **22** were synthesized as depicted in Scheme 3. Compound **23** was synthesized as shown in Scheme 4. [b-d] Cell assays were performed as described for Tables 1 and 2.

Uptake in HEK Uninduced and HEK-hLAT1 Cells

To corroborate the results of our *trans*-stimulation assay for **22** and **23**, we measured their intracellular concentrations in HEK-hLAT1 and uninduced HEK cells after a 30 min incubation time at 50 μM using LC-MS analysis (Fig. 3). We used L-phenyl-d_5-alanine (Phe-d_5) as a positive control, which could be distinguished from endogenous L-phenylalanine already present within cells. Consistent with expectations, Phe-d_5 displayed a 3.5-fold greater uptake in HEK-hLAT1 cells relative to uninduced cells. In contrast, the difference in uptake between induced and uninduced cells for **22** and **23** was much smaller (1.2–1.5-fold). The uptake of **22** in uninduced HEK cells was approximately one-tenth the value reported in MCF-7 cells (0.013 ± 0.002 vs. 0.10 ± 0.01 nmol/mg/min),[15] which is likely due to a greater expression of transporters on the latter's surface. For example, **22** was also reported to interact with organic-anion-transporting polypeptides (OATP),[15] which are expressed in cancer cells.[25] Surprisingly, compound **23** had about a 10-fold greater uptake into both induced and uninduced HEK cells compared with **22**. We currently do not have an explanation for this discrepancy; however, it is possible that compound **23** is gaining entry by a transporter that is selective against **22** or maybe **23** has some passive diffusion.

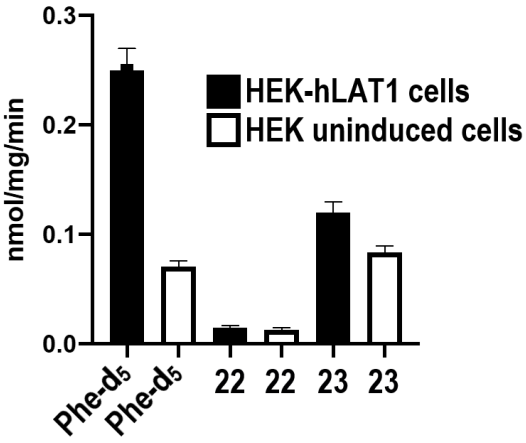


Figure 3. Uptake of Phe-d_5, **22**, and **23** in HEK-hLAT1 cells and in HEK uninduced cells (mean ± SD, n = 3) after a 30 min incubation time at 50 μM, normalized to milligrams of cell protein.

Taken together, these results indicate that if LAT1 is contributing to cell uptake for **22** or **23**, it is small in comparison with Phe-d_5. Perhaps, **22** and **23** are too large to be

accommodated in the binding site as LAT1 changes conformations from outward-facing to inward-facing. Alternatively, they may bind a different conformation (*e.g.* outward-facing conformation) so strongly that the barrier to conformational change is prohibitive.

Rat Brain Perfusion Studies

Given the surprisingly good uptake of **23** into HEK cells relative to Phe-d_5 and **22**, we wondered whether it might be BBB permeable, even if LAT1's contribution toward transport was small. Thus, **23** was tested alongside Phe-d_5 and **22** in a modified *in situ* rat brain perfusion experiment.[26] Though many techniques exist to study transport across the BBB,[27] *in situ* brain perfusion effectively measures brain uptake in live mammalian models. Also, due to direct injection into the rat carotid artery, which is flushed with perfusion buffer prior to adding compound, we would expect prodrugs such as **22** or **23** to be the major species being presented at the BBB rather than their metabolites (*i.e.* ketoprofen or alcohol **17**, respectively). For this experiment, test compounds were perfused at 500 μM for 1 min, which we found to be needed to reliably determine brain levels using our single quad LC-MS system. It is likely that at this high concentration most active transport mechanisms would be saturated, including LAT1.

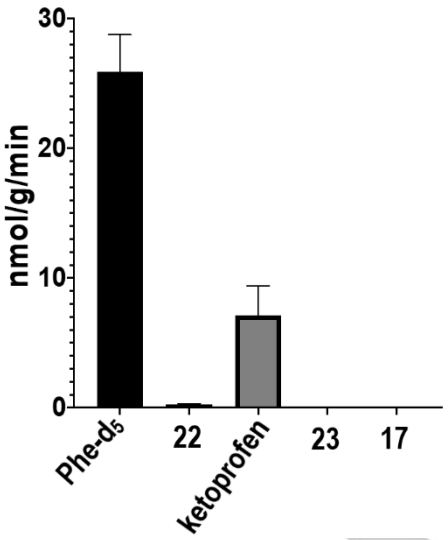


Figure 4. Rat brain uptake after perfusion of Phe-d_5, **22**, and **23** at 500 μM for 1 min, normalized to brain hemisphere mass (mean ± SD, n = 3). Brain levels of respective metabolites ketoprofen and alcohol **17** are plotted on the same graph for comparison sake. Both **23** and **17** were below our limit of detection of 0.3 nmol/g/min.

Phe-d_5 showed considerably greater rat brain uptake (26 ± 3 nmol/g/min) compared with ketoprofen-derived prodrugs **22** and **23** (Fig. 4). Uptake of **22** was at the limit of detection for our LC-MS (0.3 nmol/g/min). Consistent with what had been reported for rat brain perfusion of the para isomer **21**,[6a] we also observed extensive intrabrain metabolism of **22** to give parent drug ketoprofen (7.1 ± 2.3 nmol/g/min).[28] Compound **22** has been shown to be cleaved by esterases in various species and tissue types, including rat brain S9 fraction to give ketoprofen as the sole metabolite besides the phenylalanine-derived promoiety.[15] Assuming a direct relationship between uptake of **22** and ketoprofen brain levels, the amount of **22** transported into the rat brain was approximately one-third the value of Phe-d_5.

Unfortunately, neither **23** nor its expected metabolite **17**, were detected in rat brain (<0.3 nmol/g/min). This result contrasts with the higher uptake of **23** into HEK uninduced cells relative to Phe-d_5 and **22**. It may be that **23** is effluxed by an ABC transporter faster than uptake at the BBB. Alternatively, **23** may be entering HEK cells by transporters that are less abundant at the BBB, which could also help explain its dramatically greater cell uptake compared with **22**. Nonetheless, these findings support our interpretation of data from HEK-hLAT1 cells that **23** is not a LAT1 substrate. Though we cannot rule out the possibility of LAT1 playing a role in BBB transport of **22**, its brain levels were significantly lower than Phe-d_5. Moreover, as has been reported,[15] **22** is a substrate for the low affinity-high capacity transporter OATP2 (SLC21A5) and thus could be crossing the BBB primarily by this mechanism,[29] particularly in light of the high concentration (500 μM) we used in our perfusion experiments.

Conclusion

We have shown that both the position and orientation of a carbonyl group used to link hydrophobic substituents to an amino acid can have a considerable impact on LAT1 transport rate and potency. Additionally, we found that serine and homoserine are likely better amino acid promoieties than lysine and its homologs for use in LAT1-targeted drug delivery, as amides of the latter had poorer transport rates and were generally not substrates. However, benzoylated serine and homoserine derivatives were considerably less potent compared with meta-substituted phenylalanine analogs, providing further evidence that the carbonyl's position relative to the phenyl ring and the amino acid group are important for recognition by LAT1. Consistent with our previous findings for a methyl ester,[8] having a carbonyl group directly attached at the meta position of phenylalanine gave a better transport rate for a phenyl-substituted ester than attachment *via* the ester oxygen.

Despite the benefits of having a carbonyl directly attached at the meta position, as the size of the substituent was increased to include two aromatic rings (*i.e.* ketoprofen analog **17**), the transport rate decreased along with a dramatic increase in potency for "reverse ester" **23**. Despite previous reports that ketoprofen-tyrosine prodrugs (*i.e.*, **21** and **22**) are LAT1 substrates,[6a,15] based on our experiments using an inducible cell line, HEK-hLAT1, we found them to be potent LAT1 inhibitors and not substrates. Moreover, rat brain perfusion experiments corroborated our interpretation of cell assay data, as prodrug **22** and its analog **23**, in which the orientation of the ester group was reversed, had poor to no brain uptake, relative to L-phenyl-d_5-alanine. Taken together, we conclude that though LAT1 can transport phenylalanine derivatives with relatively small substituents, at least up to the size of a phenyl ring, larger substituents are more likely to lead to LAT1 inhibition and not substrate activity. These findings are highly relevant to LAT1-targeted treatment of both neurological diseases and cancer.

Experimental Section

This experimental section includes full details for synthesis of final products, cell assay conditions, modeling methods, and procedures for rat brain and cellular uptake studies. The following can be found in supporting information: docking poses of compounds **13** and **14** (Figure S1); docking scores of compounds from Tables 1-3 (Table S1); synthesis and summary of deuterated standards for test compounds used in LC-MS analysis of rat brain tissue samples (Table S2); ^{1}H and ^{13}C NMR spectra of newly synthesized, final products (**3b-d**, **4b**, **13**, **14**, **23**); and relative uptake of [^{3}H]-gabapentin into un-transfected, un-induced and induced HEK-hLAT1 cells in the presence and absence of LAT1 inhibitor BCH at 2 mM (Figure S2).

Ligand Docking. Molecular docking was performed using Glide from the Schrödinger suites. All ligands were docked to the recently solved cryo-EM structure of LAT1 (PDB: 6IRT).[12b] The 6IRT structure was prepared for docking with the Maestro Protein Preparation Wizard[30] under default parameters. The ligand binding site was defined based on the coordinates of LAT1 inhibitor BCH[23] from the 6IRT structure. The receptor grid for docking was generated *via* Maestro Receptor Grid Generation panel.[30] The small molecules used in molecular docking were prepared for docking using LigPrep of the Schrödinger suite.[30] The docking results were visualized *via* PyMOL.[31]

Synthesis General. Flash Column Chromatography was performed either using silica gel (porosity 60 Å, particle size: 40-63 μm, 230 x 400 mesh) from Sorbent Technologies in Chemglass columns or using a Teledyne ISCO NextGen300+ Flash Chromatography System (RediSepRf High Performance GOLD silica cartridges). Preparative HPLC performed on a Gilson PLC 2020. Column: Synergi 4μ Fusion-RP by Phenomenex, 150 x 21.2 mm, protected with a SecurityGuard PREP Cartridge, C12, 15 x 21.2 mm. Preparative HPLC methods: Each of the following methods employed isocratic elution at 20 mL/min flow rate with the indicated percentage (%) of CH_3CN in non-buffered Milli-Q deionized water (Integral 5 Water Purification System). Method A: 0% (*i.e.* water only); B: 2%; C: 5%; D: 10%; E: 30%. Compounds purified by HPLC were concentrated by lyophilization using a Labconco Freezone 2.5 Plus. LC-MS analysis was performed using an Agilent G6125 single quad ESI source and a 1260 Infinity HPLC system (G7112B Binary Pump and G7114A Dual λ Absorbance Detector). Column: Synergi 4μ Fusion-RP by Phenomenex, 150 x 4.6 mm. The following LC-MS methods (A-F) were performed using 1.0 mL/min flow rates: Method A: gradient elution, starting with 10% CH_3CN in 0.1% formic acid and ramping to 80% over 10 min. For LC-MS methods B-F, isocratic elution was performed using a mobile phase containing the following percentages (%) of CH_3CN in 0.1% formic acid. LC-MS Method: B, 5%; C, 10%; D, 20%; E, 30%; F, 50%.

^{1}H and ^{13}C NMR were recorded on an Avance III HD Bruker instrument operating at 400 and 100 MHz, respectively. Unless indicated otherwise, NMR spectra were obtained as $CDCl_3$, CD_3OD, D_2O and $(CD_3)_2SO$ solutions (reported in ppm), using residual solvent peaks in the ^{1}H and ^{13}C NMR spectra ($CDCl_3$: 7.27, 77.23 ppm; CD_3OD: 3.31, 49.15 ppm; D_2O, 4.79; and $(CD_3)_2SO$: 2.50, 39.51 ppm) as the reference standard, respectively. All *J* values are given in units of Hz. Optical rotations were measured on a Rudolph Research Autopol III polarimeter (using sodium D line, 589 nm) and $[\alpha]_D$ given in units of (degrees-mL)/(dm-g), and concentration (c) is reported in units of g/100 mL. All water used in analysis and for preparative HPLC was purified by a Milli-Q® Integral 5 Water Purification System. Melting points were obtained using a Mel-Temp apparatus and are uncorrected. Sonication was performed using a VWR Aquasonic Model 75T.

General procedure for benzoylation of BOC protected lysine homologs (2a-c): Commercially available BOC-protected lysine homologs **1a-c** (1.0 equiv) and NaOH (2.1 equiv) were dissolved with stirring in 1:1 dioxane/water (0.5 M). The solution was cooled in an ice bath, and benzoyl chloride (1.2 equiv) was added dropwise. Reaction mixture was stirred overnight at rt. Acidified the cooled reaction mixture with 3M aq. HCl. Extracted products with diethyl ether, dried ($MgSO_4$), and concentrated *in vacuo*. Crude products were taken forward to the next step without purification. Yields: 95-100%.

General procedure for BOC deprotection to give benzoylated lysine homologs (3a-c): BOC-protected homologs **2a-c** (2.0 mmol, 1.0 equiv) were stirred overnight at rt with triethylsilane (3.0 equiv) and 1:1 TFA (10 equiv)/DCM. Reaction mixture was concentrated *in vacuo* and then purified by preparative HPLC (Method B). **3a:** Yield: 56 mg (14%); >98% purity by LC-MS (254 nm, Method B), ^{1}H NMR ($(CD_3)_2SO$) δ 8.72 (s, 1H), 7.84 (d, *J* = 7Hz, 2H), 7.58-7.43 (m, 3H), 4.09 (m, 1H), 3.58 (m, 2H); in accordance with reference.[32] m/z (ESI-pos) M+1 = 209.1. **3b**: Yield: 54 mg (12%), >98% purity by LC-MS (254 nm, Method C), mp 210-213 °C, $[\alpha]_D^{24}$ +14° (c 0.45, 1M aq. HCl), ^{1}H NMR (D_2O + 5%DCl) δ 7.01 (m, 2H), 6.88 (m, 1H), 6.78 (m, 2H), 3.48 (t, *J* = 6Hz, 1H), 2.89 (t, *J* = 6Hz, 2H), 1.59 (m, 2H); ^{13}C NMR ($(CD_3)_2SO$ + 5% DCl) δ 170.9, 168.9, 134.1, 131.6, 128.5, 127.5, 50.1 (m), 35.5 (m), 30.0. m/z (ESI-pos) M+1=223.1. **3c**: Yield: 280 mg, (60%), >98% purity LC-MS (254 nm, Method C), mp 229-231°C dec, $[\alpha]_D^{24}$ +19° (c 0.51, 1M aq. HCl), ^{1}H NMR (D_2O) δ 7.72 (m, 2H), 7.58 (m, 1H), 7.49 (m, 2H), 3.76 (m, 1H), 3.41 (m, 2H), 1.93 (m, 2H), 1.70 (m, 2H); ^{13}C NMR (D_2O, CH_3CN added as internal standard) δ 172.6, 171.6, 134.1, 132.6, 129.3, 127.5, 53.3, 39.5, 27.7, 24.7. m/z (ESI-pos) M+1=237.1.

Methyl *N*6-benzoyl-*N*2-(*tert*-butoxycarbonyl)-L-lysinate (2d): Commercially available methyl (tert-butoxycarbonyl)-L-lysinate hydrochloride **1d** (0.35 g, 1.2 mmol) and DMAP (14 mg, 0.12 mmol) were dissolved in DCM (4 mL) and stirred at 0 °C. *N*-Ethyl-*N*-isopropylpropan-2-amine (0.52 mL, 2.5 mmol) was added and the reaction was left to stir for 10 min, followed by addition of benzoyl chloride (0.21 mL, 1.8 mmol) all-at-once. Reaction mixture was stirred for 1 h at rt. Then, the reaction was acidified with 1M aq. HCl (10 mL), extracted with diethyl ether (2 x 10 mL), dried ($MgSO_4$) and filtered, and concentrated *in vacuo*. Crude product was carried forward to the next step without purification. Yield: 0.475 g (100%).

***N*6-Benzoyl-L-lysine (3d):** Saponification of the methyl ester functional group in **2d** was achieved using LiOH in a manner similar to that described previously.[33] Crude product was carried directly forward into BOC deprotection by stirring with 1:1 TFA/DCM (5 mL) overnight at rt. Removed solvents *in vacuo*. Desired product **3d** was purified by preparative HPLC (Method D). Yield: 0.12 g (41%), >98% purity LC-MS (254 nm, Method A), mp 234-240 °C dec, $[\alpha]_D^{24}$ +19.2° (c 0.89, 1M aq. HCl), ^{1}H NMR (D_2O) δ 7.59 (m, 2H), 7.46 (m, 1H), 7.37 (m, 2H), 3.98 (m, 1H), 3.29 (m, 2H), 1.88 (m, 2H), 1.56 (m, 2H), 1.39 (m, 2H). ^{13}C NMR ($(CD_3)_2SO$ + 5% DCl) δ 173.4, 173.0, 135.4, 133.9, 131.5, 129.9, 54.9, 42.5, 31.6, 30.0, 24.2. m/z (ESI-pos) M+1=251.1.

General procedure for benzoylation of serine and homoserine (4a-b): L-Serine or L-homoserine (1 equiv) were stirred with TFA (1.7M) at 0 ºC for 15 min. Then, benzoyl chloride (1.5 equiv) was added all-at-once and the mixture was stirred vigorously overnight at rt. The reaction mixture was diluted with cold diethyl ether (50 mL), and the suspension was filtered and the resulting solids were dried under high vacuum. The crude product was purified by preparative HPLC (Method C). **4a**: Yield: 65 mg (24%), >95% purity by LC-MS (254 nm, Method C), ^{1}H NMR ($(CD_3)_2SO$ + 5%DCl) δ 8.13-7.36 (m, 5H), 4.68 (m, 2H), 4.48 (m, 1H). Our ^{1}H NMR was consistent with the reported spectrum.[34] m/z (ESI-pos) M+1=210.1. **4b**: Yield: 50 mg (16%), >98% purity by LC-MS (254 nm, Method B), mp 198-200°C, $[\alpha]_D^{24}$ +37° (c 0.66, 1M aq. HCl), ^{1}H NMR (D_2O + 5%DCl) δ 7.72 (m, 2H), 7.43 (m, 1H), 7.27 (m, 2H), 4.26 (m, 2H), 4.08 (m, 1H), 2.25 (m, 2H); ^{13}C NMR (D_2O, acetone added as internal standard) δ 172.0, 168.9, 134.6, 130.0, 129.5, 129.4, 61.4, 51.0, 29.3. m/z (ESI-pos) M+1=224.1.

Phenyl 3-bromobenzoate (5): To a stirred mixture of DMAP (0.12 g , 1.0 mmol), 3-bromobenzoic acid (2.0 g, 10 mmol), phenol (0.94 g, 10 mmol), and THF (3 mL) was added N,N'-diisopropylcarbodiimide (1.3 g, 10 mmol)

at 0 °C. Reaction mixture was left to stir overnight at rt. Then, the mixture was suspended in 1:1 DCM/hexanes (10 mL). The suspension was filtered to remove insoluble diisopropyl urea and the filtrate was concentrated *in vacuo*. Mixture was purified by flash chromatography (10% EtOAc/hexanes). Yield: 2.1 g (74%). ^{1}H NMR ($CDCl_3$) δ 8.37 (s, 1 H), 8.16 (d, *J* = 8Hz, 1H), 7.79 (d, *J* = 8Hz, 1H), 7.44 (m, 3H), 7.31 (m, 1H), 7.23 (m, 2H).

Phenyl (*S*)-3-(3-(*tert*-butoxy)-2-((*tert*-butoxycarbonyl)amino)-3-oxopropyl)benzoate (7): The title compound was prepared from *tert*-butyl (*R*)-2-((*tert*-butoxycarbonyl)amino)-3-iodopropanoate[8] (1.9 g, 5.0 mmol) and aryl bromide **5** (1.4 g, 5.0 mmol) using Negishi coupling conditions that we previously described.[8] Crude was partially purified *via* flash chromatography (10% EtOAc/hexanes), and then carried forward without further purification. Yield: 1.0 g (45%).

(*S*)-2-((*tert*-Butoxycarbonyl)amino)-3-(3-hydroxyphenyl)propanoic acid (8c): BOC protection of *meta*-L-tyrosine **8b** (1.0 g, 5.5 mmol) was performed in a manner similar to that previously described for L-DOPA.[35] Yield: 1.2 g (80%). Product was carried to the next step without purification.

(*S*)-2-Amino-3-(3-(phenoxycarbonyl)phenyl)propanoic acid hydrochloride (13): BOC deprotection of phenyl (*S*)-3-(3-(*tert*-butoxy)-2-((*tert*-butoxycarbonyl)amino)-3-oxopropyl)benzoate **7** (1.0 g, 2.3 mmol) was performed using TFA/Et_3SiH in a manner as we previously described.[36] Product was purified *via* preparative HPLC (Method E) and converted to an HCl salt by freeze drying in the presence of aqueous HCl. Yield: 20 mg (3%), >98% purity through analysis of LC-MS (254 nm, Method E), mp 208-211 °C dec, $[\alpha]_D^{24}$ +2.7° (c 0.38, 1M aq. HCl), ^{1}H NMR (D_2O + 5% DCl) δ 8.30-8.37 (m, 2H), 7.79-7.91 (m, 2H), 7.73 (m, 2H), 7.59 (m, 1H), 7.47 (m, 2H), 4.56 (t, *J* = 7Hz, 1H), 3.57 (m, 2H); ^{13}C NMR (D_2O + 5% DCl, and 5% CD_3CN as internal standard) δ 171.5, 167.4, 151.6, 136.3, 136.2, 131.8, 130.9, 130.7, 130.4, 127.5, 122.7, 119.9, 54.7, 36.4. m/z (ESI-pos) M + 1 = 286.1.

(*S*)-2-Amino-3-(3-(benzoyloxy)phenyl)propanoic acid hydrochloride (14): The title compound was prepared from *meta*-L-tyrosine (0.15 g, 0.82 mmol) using reaction conditions similar to those of **4a-b**. After removal of volatiles by concentration *in vacuo*, the product was converted to its HCl salt by dissolution in *p*-dioxane (2 mL) and 4M HCl in dioxane (1.2 mL). Concentrated *in vacuo* and then triturated resulting solids with diethyl ether. The solids were filtered, rinsing with diethyl ether and dried under high vacuum. Yield: 71 mg (30%), >98% purity by LC-MS (254 nm, Method D), mp 183-187 °C dec, $[\alpha]_D^{24}$ 0° (c 0.091, DMSO), ^{1}H NMR (D_2O) δ 8.35 (m, 2H), 7.94 (m, 1H), 7.79 (m, 2H), 7.70 (m, 1H), 7.49 (m, 1H), 7.38 (m, 2H), 4.51 (m, 1H), 3.56 (dd, *J* = 6, 15Hz, 1H), 3.43 (dd, *J* = 8, 15Hz, 1H); ^{13}C NMR (D_2O + 5% DCl, CD_3CN added as internal standard) δ 171.8, 168.2, 151.9, 137.3, 135.6, 131.7, 131.0, 130.0, 129.6, 128.7, 123.7, 122.4, 120.1, 54.9, 36.3. m/z (ESI-pos) M+1=286.1.

(*S*)-2-Amino-3-(4-(benzoyloxy)phenyl)propanoic acid hydrochloride (15): The title compound was prepared from L-tyrosine (1.0 g, 5.5 mmol) according to the procedure for compound **14**, and its synthesis has previously been described.[17] Yield: 430 mg (27%), >98% purity by LC-MS (254 nm, Method D). ^{1}H NMR was consistent with the previously reported spectrum.[17] ^{1}H NMR (CD_3OD) δ 8.19 (d, *J* = 8Hz, 2H), 7.72 (m, 1H), 7.59 (m, 2H), 7.43 (m, 2H), 7.28 (m, 2H), 4.32 (m, 1H), 3.14-3.34 (m, 2H, overlaps with CHD_2OD). m/z (ESI-pos) M+1=286.1.

(3-(1-Hydroxypropan-2-yl)phenyl)(phenyl)methanone (17): The title compound was prepared as previously described,[18] however in our hands the yield was considerably lower than what had been reported. Purified product using an ISCO flash chromatography system (40 g silica cartridge), eluting with a gradient of 10-50% EtOAc/hexanes. Yield: 0.49 g (26%). ^{1}H NMR (CD_3OD) δ 8.16 (m, 2H), 7.68 (m, 1H), 7.55 (m, 2H), 7.39 (d, *J* = 8Hz, 2H), 7.24 (d, *J* = 9Hz, 2H), 4.29 (m, 1H), 3.37 (dd, *J* = 6, 16Hz, 1H), 3.28 (m, 3H), 3.17 (dd, *J* = 8, 15Hz, 1H).

(S)-3-(2-Amino-2-carboxyethyl)benzoic acid hydrochloride (18): The title compound was prepared from (S)-2-amino-3-(3-cyanophenyl)propanoic acid (5.0 g, 26 mmol) using a procedure described by Peura[11b]. Yield: 5.1 g (79%).

3-(((1*R*,4'*S*,5*S*)-5'-Oxo-9λ^4-boraspiro[bicyclo[3.3.1]nonane-9,2'-[1,3,2]oxazaborolidin]-4'-yl)methyl)benzoic acid (19): The following synthesis is a modification of a procedure described by Peura.[11b] Charged a dry round bottom flask and stir bar with *meta*-carboxyl phenylalanine hydrochloride **18** (4.0 g, 16 mmol), anhyd. DMF (40 mL), and anhyd. pyridine (2.6 mL, 33 mmol). Cooled flask in an ice bath and added 9-methoxy-9-borabicyclo[3.3.1]nonane (1M in THF, 16 mL, 16 mmol) to the stirred solution, dropwise. Allowed reaction to slowly warm to rt and stirred overnight. Transferred mixture to a separatory funnel using EtOAc (150 mL). Washed organic phase with 1M aq. $KHSO_4$ (50 mL). Re-extracted aqueous phase with EtOAc (50 mL). Combined organic phases were washed with water (2 x 50 mL), brine (50 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. Heated the crude product with EtOAc (50 mL) to near boiling, using a spatula to break up the larger pieces of solid. Allowed the suspension to cool to rt before filtering. Dried resulting white solid under high vacuum overnight. Yield: 3.7 g (69%). ^{1}H NMR ($(CD_3)_2SO$) δ 12.9 (br s, 1H), 7.97 (s, 1H), 7.82 (d, *J* = 6Hz, 1H), 7.60 (d, *J* = 6Hz, 1H), 7.44 (m, 1H), 6.44 (m, 1H), 5.92 (m, 1H), 3.87 (m, 1H), 3.24 (m, 1H), 2.99 (m, 1H), 1.27-1.84 (m, 12H), 0.45 (m, 2H).

2-(3-Benzoylphenyl)propyl 3-(((1*R*,4'*S*,5*S*)-5'-oxo-9λ^4-boraspiro[bicyclo[3.3.1]nonane-9,2'-[1,3,2]oxazaborolidin]-4'-yl)methyl)benzoate (20): Charged a dry round bottom flask and stir bar with boroxazolidone **19** (569 mg, 1.73 mmol), alcohol **17** (415 mg, 1.73 mmol), DMAP (21 mg, 0.17 mmol) and anhydrous THF (5 mL). The stirred mixture was cooled in an ice bath under argon. N,N'-Diisopropylcarbodiimide (0.54 mL, 3.5 mmol) was added dropwise. The mixture was allowed to warm to rt, and stirring was continued overnight. The reaction mixture was concentrated *in vacuo*. The resulting residue was suspended in DCM (2-3 mL), filtered to remove diisopropylurea, and then purified by an ISCO flash chromatography system (40 g silica cartridge), eluting with a gradient of 20%-100% EtOAc/hexanes. Yield: 605 mg (64%). ^{1}H NMR ($CDCl_3$) δ 7.93 (m, 1H), 7.80-7.90 (m, 2H), 7.76-7.80 (m, 2H), 7.59-7.68 (m, 1H), 7.36-7.59 (m, 7H), 5.45 (m, 1H), 4.28-4.58 (m, 2H), 4.02-4.28 (m, 2H), 3.14-3.52 (m, 3H), 1.06-1.99 (m, 15H), 0.63 (s, 1H), 0.25 (d, *J* = 17 Hz, 1H).

(2*S*)-3-(3-((2-(3-Benzoylphenyl)propanoyl)oxy)phenyl)-2-((*tert*-butoxycarbonyl)amino)propanoic acid (22-BOC): A solution of ±ketoprofen acid chloride **16** [prepared by refluxing thionyl chloride with ±ketoprofen as described by Napoleon[37] (444 mg 1.63 mmol)] dissolved in DCM (1 mL) was added dropwise to a stirred mixture of BOC-protected *meta*-L-tyrosine **8c** (458 mg, 1.63 mmol), Et_3N (0.69 mL, 4.9 mmol), and DMAP (20 mg, 0.16 mmol) in DCM (1 mL) at 0 °C under argon. The reaction was allowed to warm to rt slowly and stirring was continued overnight. The reaction mixture was then cooled in an ice bath, and 1M aq. HCl (10 mL) was carefully added. Extracted product with DCM (2 x 10 mL). Dried combined organic phases ($MgSO_4$), filtered and concentrated. Purified using an ISCO flash chromatography system (12 g silica cartridge) eluting with a gradient of 10%-50% EtOAc/hexanes (both mobile phases containing 1% HOAc). Dried product under high vacuum overnight. Yield: 382 mg (45%). ^{1}H NMR ($CDCl_3$) δ 7.86 (m, 1H), 7.82 (m, 2H), 7.70 (m, 1H), 7.58-7.65 (m, 2H), 7.46-7.52 (m, 3H), 7.28 (m, 1H), 7.04 (m, 1H), 6.92 (m, 1H), 6.84 (m, 1H), 4.99 (m, 1H), 4.58 (m, 1H), 4.03 (q, *J* = 7Hz, 1H), 3.13 (m, 2H), 1.64 (d, *J* = 7Hz, 3H), 1.39 (br s, 9H).

(2S)-2-Amino-3-(3-((2-(3-benzoylphenyl)propanoyl)oxy)phenyl)propanoic acid hydrochloride (22): The title compound has also been synthesized by an alternate route.[15] To **22-BOC** (380 mg, 0.74 mmol) was added HCl (4M in dioxane, 2.8 mL, 11 mmol), and the mixture was stirred for 30 min at rt. Solvent was removed *in vacuo*, and the resulting powder was triturated with diethyl ether and filtered, rinsing several times with diethyl ether. Product was

dried overnight under high vacuum. Yield: 260 mg (79%), >98% purity by LC-MS (254 nm, Method F). ^{1}H NMR was consistent with the previously reported spectrum.[15] ^{1}H NMR ($(CD_3)_2SO$) δ 13.7 (br s, 1H), 8.42 (br s, 3H), 7.50-7.90 (m, 9H), 7.38 (m, 1H), 7.20 (m, 1H), 7.03 (m, 2H), 4.26 (m, 1H), 4.15 (m, 1H), 3.15 (m, 2H), 1.55 (d, *J* = 7Hz, 3H). *m/z* (ESI-pos) M + 1 = 418.2.

(2*S*)-2-Amino-3-(3-((2-(3-benzoylphenyl)propoxy)carbonyl)phenyl)propanoic acid hydrochloride (23): To a solution of boroxazolidone **20** (555 mg, 1.01 mmol) in THF (5.5 mL) was added TBAF (1M in THF, 4.0 mL, 4.0 mmol), and the mixture was stirred overnight at rt. Consistent with previously described deprotection conditions for amino acid-containing boroxazolidones,[19] water (10 mL) was added and the mixture was stirred for 5 min. Acidified the mixture to pH 3-4 using neat formic acid (1-2 mL). Concentrated mixture *in vacuo*, and then diluted the resulting residue to approximately 30 mL total volume using 1:1 CH_3CN/water. Additional formic acid (1-2 mL) was added and the mixture was sonicated to aid dissolution. Undissolved solids were removed by a 0.45 μm syringe filter prior to purification by preparative HPLC (gradient elution of 10% CH_3CN in water ramping to 80% over 15 min). Product-containing fractions were lyophilized. Desired product was converted to its HCl salt by suspending the lyophilized solids in dioxane (3 mL) and adding 4M HCl in dioxane (0.25 mL). After sufficient mixing and sonication, the suspension was diluted with diethyl ether (10 mL) and the solids were filtered, rinsing several times with diethyl ether. Dried solids under high vacuum overnight. Yield: 179 mg (38%). >98% purity by LC-MS (254 nm, Method E). mp 166-172 °C dec, $[\alpha]_D^{24}$ +8.7° (c 1.1, DMSO). ^{1}H NMR (CD_3OD) δ 7.92 (s, 1H), 7.87 (d, *J* = 8Hz, 1H), 7.70-7.80 (m, 3H), 7.58-7.68 (m, 3H), 7.41-7.58 (m, 5H), 4.62 (d, *J* = 6 Hz, 2H), 4.27 (m, 1H), 3.36 (m, 2H), 3.21 (dd, *J* = 8, 15 Hz, 1H), 1.44 (d, *J* = 7 Hz, 3H). ^{13}C NMR ($(CD_3)_2SO$ + 5% DCl) δ 196.0, 170.0, 165.6, 143.8, 137.3, 137.1, 135.9, 134.6, 132.8, 131.9, 130.4, 130.0, 129.7, 129.1, 128.8, 128.7, 128.5, 128.2, 127.9, 69.3, 52.9, 38.4, 35.3, 17.7. *m/z* (ESI-pos) M + 1 = 432.2.

Cell culture and characterization. TREx HEK-hLAT1 (XenoPort, Inc., Santa Clara, CA)[20b] is a tetracycline inducible cell line encoding both LAT1 and 4F2hc (SLC3A2). The cell line was previously validated for uptake of known LAT1 substrates.[8] Cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) H-21 media supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 units/mL streptomycin, 1 μg/mL Fungizone, 2 mM L-glutamine and 3 μg/mL blasticidin. The incubation conditions were 37 °C and 5% CO_2. The maximum cell passage used in this study was 15. Uptake of [^{3}H]-gabapentin into induced HEK-hLAT1 cells (supporting information: Figure S2) was verified prior to each cell assay.

***cis*-Inhibition assay and IC_{50} determinations.** HEK-hLAT1 cells were seeded at 200,000 cells/well and grown on poly-D-lysine coated 24-well plates in DMEM medium to achieve at least 90% confluence after 48 h. As previously described,[8] cells were treated with 1 μg/mL doxycycline and 2 mM sodium butyrate for 24 h before the uptake assay. During uptake assays, cells were first washed with sodium-free choline buffer (140 mM choline chloride, 2 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, and 1 M Tris) and incubated for 10 min in the same buffer. After that, the buffer was removed and replaced with uptake buffer (sodium-free choline with 6 nM of [^{3}H]-gabapentin and inhibitors at appropriate concentrations). The primary screen was done at 200 μM. The IC_{50} determination experiments were performed with concentrations from 0.1 to 500 μM. Uptake was performed at 37 °C for 3 min, and then terminated by washing the cells twice with ice-cold choline buffer. Cells were lysed *via* lysis buffer (800 μL) (0.1 N NaOH and 0.1% SDS) and allowed to sit at rt for 3 h. Intracellular radioactivity was determined by scintillation counting on a LS6500 Scintillation Counter (Beckman Coulter). IC_{50} and standard deviation of each compound were analyzed using GraphPad Prism version 5.0. %[^{3}H]-Gabapentin uptake at each concentration was normalized relative to %inhibition by BCH[23] at 2 mM, which was set to 100% inhibition.

***trans*-Stimulation assay**. HEK-hLAT1 cells were seeded at 200,000 cells/well and grown on poly-D-lysine coated 24-well plates in DMEM medium to achieve at least 90% confluence after 48 h. As previously described,[8] cells were treated with 1 μg/mL doxycycline and 2 mM sodium butyrate for 24 h before the uptake assay. During uptake assays, cells were first washed with sodium-free choline buffer (140 mM choline chloride, 2 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, and 1 M Tris) and incubated for 10 min in the same buffer. After that, the buffer was removed and replaced with preincubation buffer (sodium-free choline with 6 nM of [^{3}H]-gabapentin) and pre-incubated for 30 min. The pre-incubation buffer was removed, and cells were washed twice with sodium-free choline buffer. The *trans*-stimulation buffer containing sodium-free choline buffer and tested compounds at 200 μM was then added. An aliquot of the *trans*-stimulation buffer was collected at 3 min for extracellular radioactivity measurement. The extracellular radioactivity was determined by scintillation counting on a LS6500 Scintillation Counter (Beckman Coulter). Efflux of [^{3}H]-gabapentin was calculated at 3 min after adding test compound. %Efflux was normalized relative to L-Phe, which had an efflux rate of 2.7 ± 0.3 fmol/min, from an average of seven experiments.

HEK-hLAT1 Cell Uptake Studies. HEK-hLAT1 cells were seeded at 300,000 cells/well and grown on poly-D-lysine coated 6-well plates in DMEM medium to achieve at least 90% confluence after 48 h. As previously described,[8] cells were treated with 1 μg/mL doxycycline and 2 mM sodium butyrate for 24 h before the uptake assay. During uptake assays, cells were first washed with sodium-free choline buffer (140 mM choline chloride, 2 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, and 1 M Tris) and incubated for 10 min in the same buffer. After that, the buffer was removed and replaced with uptake buffer (2.5 mL per well) (sodium-free choline with tested compounds at 50 μM). Uptake was performed at 37 °C for 30 min, and then terminated by washing the cells twice with ice-cold choline buffer. Acetonitrile (500 μL) was added to each well and pipetted up and down ~ 20 times. The acetonitrile cell extracts were transferred to 1.5 mL microcentrifuge tubes and evaporated by blowing nitrogen. Once fully evaporated, the cell extracts were frozen at -20 °C. Remaining cells in each well were lysed *via* lysis buffer (800 μL) (0.1 N NaOH and 0.1% SDS) and allowed to sit at rt for 3 h. An aliquot of cell lysates was collected for protein quantification *via* a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA).

Cell extract was diluted to 1.0 mL with either 0.1% formic acid (for Phe-d_5) or with 1:1 CH_3CN/water (for compounds **22** and **23**). Samples were then passed through a syringe filter (0.45 μm) and analyzed directly by single quad LC-MS in selected ion monitoring (SIM) mode (see instrument and column details provided in "Synthesis General" section, above) eluting with either isocratic 0.1% formic acid for 8 min, then ramping to 80% CH_3CN + 20% 0.1% formic acid over 4 min, and holding for 2 min for analysis of Phe-d_5; or eluting with isocratic 40% CH_3CN + 60% 0.1% formic acid for 7 min, then ramping to 80% CH_3CN + 20% 0.1% formic acid over 1 min, and holding for 2 min for analysis of **22** and **23**. Peak areas for parent ions (M+1) were converted to amount (nmol) using standard curves generated over a 20-fold concentration range for analyte in the presence of cell extract. Cell uptake rate (nmol/min/mg) was calculated by dividing amount of test compound (nmol) by cell uptake time (30 min) and normalized to amount of protein (mg), as determined above. All cell uptake experiments were performed in triplicate for each compound and analysis by LC-MS was also in triplicate.

***In Situ* Rat Brain Perfusion Technique.** Laboratory rats (*Rattus norvegicus*) of the Long-Evans strain, ranging in age from 6-96 weeks (average was about 72 weeks) and of both sexes, were used in all brain perfusion experiments, similar to a previously described method.[26] Rats were anesthetized *via i.p.* injections using a Ketamine:Xylazine aqueous solution (88 mg/kg : 30 mg/kg). During the procedure, rats showed slowed, but normal respiratory activity. A cannula with an internal diameter of 0.159 mm was custom-made from 30-gauge PTFE tubing. The cannula was pre-filled with a heparin solution (1 mg/mL, IU≥100/mg) before it was inserted

into the left carotid artery and secured with surgical silk. The chest cavity was opened, and the ventricles were severed to stop systemic circulation. Immediately, the brain was perfused at 10.5 mL/min for 1 min 20 sec with a standard perfusion buffer[26] at 37 °C: 128 mM NaCl, 24 mM $NaHCO_3$, 4.2 mM KCl, 2.4 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 0.9 mM $MgCl_2$. Perfusion buffer was oxygenated using 95% O_2 + 5% CO_2 for 10 min prior to use. D-Glucose was added immediately before perfusion to give a concentration of 9 mM. Then, test compound dissolved in perfusion buffer (500 μM) was perfused for 1 min 15 sec (15 sec was the time required for compound to reach the carotid artery *via* a peristaltic pump (Control Company, model 3386), as determined by observing the movement of colored solutions). Lastly, capillaries were flushed with perfusion buffer for 1 min 20 sec. The brain was then extracted, hemispheres were separated, and the left brain hemisphere was frozen using liquid nitrogen and stored in a -30 °C freezer prior to analysis.

Rat Brain Homogenization. Test compounds were extracted from brain hemisphere (after recording its mass) using a homogenization technique modified from the literature.[9b] Left brain hemisphere was added to a 50 mL centrifuge tube on ice. The brain hemisphere was spiked with a deuterated isotopomer (synthesis of deuterated analogs of test compounds are provided in supporting information) or structurally similar analog of the test compound (Table S2, supporting information; 2.5 mL, 2 μM in 20% CH_3CN/H_2O). In 10 sec pulses, each hemisphere was pulverized using a Fisher 850 homogenizer (speed 16,000). Then, a 10% aqueous trichloroacetic acid solution (2 mL) was added to precipitate proteins. The mixture was vortexed for 2 min and placed on ice for 10 min. The homogenate was centrifuged at 7,500g for 10 min at 7 °C using a Beckman Avanti J-25 high speed centrifuge. The supernatant was removed for LC-MS analysis using a Pasteur pipette.

Rat Brain LC-MS Analysis. The supernatant from tissue homogenization was freeze dried to concentrate and then the residue was diluted with 1:1 CH_3CN/H_2O (1 mL). The mixture was then filtered through a 0.2 μm syringe filter and analyzed by single quad LC-MS in selected ion monitoring (SIM) mode (instrument and column details provided in "Synthesis General" section, above; masses of analyte ions are summarized in Table S2, supporting information). Brain uptake (nmol/g/min) was calculated by taking the ratio of a test compound's peak area relative to the corresponding deuterated internal standard's peak area and multiplying by the amount (nmol) of internal standard added prior to tissue homogenization. The amount (nmol) of test compound was then normalized to mass of brain hemisphere (g) and perfusion time (min), after subtracting time required for compound to move from pump to carotid artery. All brain perfusion experiments were performed in triplicate for each compound and analysis by LC-MS was also in triplicate.

Abbreviations Used

LAT1, L-type amino acid transporter 1; BCH, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid; DIC, *N*, *N*'-diisopropylcarbodiimide; P(*o*-tolyl)$_3$, tri(*o*-tolyl)phosphine; Pd_2dba_3, tris(dibenzylideneacetone)dipalladium(0); 9-BBN, 9-borabicyclo[3.3.1]nonane; DMEM, Dulbecco's Modified Eagle Medium; HEK-hLAT1, human embryonic kidney cell line inducible for human L-type amino acid transporter 1; MCF-7, Michigan Cancer Foundation-7 breast cancer cell line; OATP, organic-anion-transporting polypeptides; cryo-EM, cryogenic electron microscopy; ABC, ATP-binding cassette.

Ethical Statement

All animals were maintained following the Guide for the Care and Use of Laboratory Animals. All procedures were conducted in accordance with Animal Care Protocol #190226, which was approved by the University of Nebraska at Kearney Animal Care and Use Committee.

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Conflict of Interest

The authors declare no competing financial interest.

Keywords: amino acids • blood-brain barrier • drug delivery • membrane proteins • prodrugs

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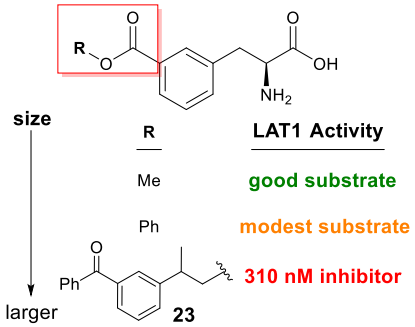
Entry for the Table of Contents



Larger amino acids were inhibitors of the L-type amino acid transporter 1 (LAT1) and had poor rat brain uptake. Small- to medium-sized esters of *meta*-carboxyl L-phenylalanine were substrates. However, larger ester substituents caused a decrease in LAT1 transport rate and an increase in potency of inhibition (**23**, IC_{50} = 0.31 ± 0.1 μM).

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