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LAT1 activity of carboxylic acid bioisosteres: Evaluation of hydroxamic acids as substrates

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ABSTRACT

Large neutral amino acid transporter 1 (LAT1) is a solute carrier protein located primarily in the blood–brain barrier (BBB) that offers the potential to deliver drugs to the brain. It is also up-regulated in cancer cells, as part of a tumor's increased metabolic demands. Previously, amino acid prodrugs have been shown to be transported by LAT1. Carboxylic acid bioisosteres may afford prodrugs with an altered physicochemical and pharmacokinetic profile than those derived from natural amino acids, allowing for higher brain or tumor levels of drug and/or lower toxicity. The effect of replacing phenylalanine's carboxylic acid with a tetrazole, acylsulfonamide and hydroxamic acid (HA) bioisostere was examined. Compounds were tested for their ability to be LAT1 substrates using both *cis*-inhibition and *trans*-stimulation cell assays. As HA-Phe demonstrated weak substrate activity, its structure–activity relationship (SAR) was further explored by synthesis and testing of HA derivatives of other LAT1 amino acid substrates (i.e., Tyr, Leu, Ile, and Met). The potential for a false positive in the *trans*-stimulation assay caused by parent amino acid was evaluated by conducting compound stability experiments for both HA-Leu and the corresponding methyl ester derivative. We concluded that HA's are transported by LAT1. In addition, our results lend support to a recent account that amino acid esters are LAT1 substrates, and that hydrogen bonding may be as important as charge for interaction with the transporter binding site.

Large neutral amino acids such as tyrosine, tryptophan, leucine, isoleucine, phenylalanine, and methionine are actively transported across cell membranes by LAT1.[1–6] Additionally, it transports amino acid-containing drugs such as gabapentin,[7,8] melphalan,[9] L-DOPA[10,11] and baclofen[12] across the blood–brain barrier (BBB). LAT1 is a sodium-independent heterodimeric membrane protein found mainly in the brain, thymus, testis, placenta, spleen, and skeletal muscle. Much of its appeal as a targeted drug delivery mechanism is due to its relative high abundance at the BBB versus other tissues (>100× BBB selective).[2,13] Besides being an instrument for CNS delivery, it has also been shown that LAT1 is up-regulated in many cancer types, including prostate,[14] esophageal,[15] colorectal,[16] gastric,[17] and non-small-cell lung cancer (NSCLC).[18] Furthermore, it has been demonstrated that cancer growth can be inhibited by blocking LAT1[19–23] which is consistent with a cancer cell's increased nutritional requirements. Thus, drugs that are able to mimic naturally-occurring amino acids (e.g., gabapentin) or prodrugs containing LAT1 recognition elements[24–28] may have far-reaching utility for treating CNS diseases and cancer.

Another advantage favoring LAT1 for drug delivery is that it is relatively tolerant to substrate structural modifications.[4,29] For example, it has been shown that in addition to hydrophobic natural α-amino acids, some β and γ amino acids (e.g., gabapentin) are also transported by LAT1.[8,30–32] Despite some flexibility in the presentation of the amine and carboxylic acid functional groups, it has been maintained that both of these functional groups are essential for transporter recognition.[4,24,33] The primary evidence supporting a carboxylic acid requirement has been centered on observations that replacement with esters and a closely related sulfonic acid resulted in loss of activity.[4,24,29] However, many of the traditional carboxylic acid bioisosteres[34,35] have apparently not been explored. Lately, this story has become further convoluted as

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Nagamori et al. reported that several carboxylic esters and a hydroxamic acid derivative of L-leucine were LAT1 substrates.[32] Their conclusions, based in part on a *trans*-stimulation assay,[36] contradicted previous reports that esters do not bind LAT1.[4,24,37]

Our group had been exploring carboxylic acid bioisosteres as LAT1 substrates, including hydroxamic acids[38,39] as part of our ongoing effort to better understand LAT1 SAR[40] prior to the recent publication by Nagamori.[32] We have been focused on modifying the carboxylic acid rather than the amine due to the potential metabolism and toxicity[41] liabilities of the former. Besides contributing to the knowledge of LAT1 SAR, replacing the carboxylic acid functional group has the potential for altering the pharmacokinetics of prodrugs[34,35] intended for LAT1 transport. Moreover, learning what functional groups may serve as surrogates for the amino acid carboxyl opens up many possibilities for the design of drugs that might benefit from this delivery mechanism.

Though numerous carboxylic acid bioisosteres have been described,[34,35,42,43] we choose to prioritize the acylsulfonamide, tetrazole, and hydroxamic acid functionalities (Table 1). These groups were selected due to their comparable pK_a and/or structural similarity to the carboxylic acid functional group,[34] and they have previously demonstrated biological activity in other series.[44–46] The acylsulfonamide (or sulfonimide) has a similar geometry and pK_a as a carboxylic acid, and it was successfully applied as a cysteinyl leukotriene (LTE4) receptor antagonist that demonstrated greater activity than the parent carboxylic acid.[47] Acylsulfonamides were also chosen because they are convenient to synthesize from the corresponding carboxylic acid. Though slightly larger than a carboxylic acid,[48] tetrazoles faithfully reproduce their trigonal planar shape and acidity (pK_a: 4.5–4.9), as the tetrazole anion is stabilized by delocalization. The tetrazole group, which is present in the orally active angiotensin II receptor antagonist Losartan,[46] has the potential to improve oral bioavailability of resulting prodrugs relative to parent carboxylic acid. Hydroxamic acids (pK_a: 8–9) are known primarily for their metal-chelating abilities; and though dramatically less acidic than the previous two bioisosteres, they have been reported as MAP/ERK kinase inhibitors where they displayed similar ADME properties to carboxylic acids.[44,49] However, use of hydroxamic acids could be limited by hydrolysis to parent carboxylic acid in vivo.[50]

Compounds were evaluated in both *cis*-inhibition and *trans*-stimulation assays using HEK cells engineered to overexpress human LAT1.[51,52] *cis*-Inhibition studies were used to identify LAT1 transport inhibitors, which may be potential substrates. However, to more directly identify substrates, we performed a *trans*-stimulation experiment[36] which exploits LAT1's alternating access mechanism[8,53] by loading cells with a radiolabeled substrate followed by incubation with extracellular test compound. The exchange efflux rate of the radiolabel in the presence of a test compound is compared with the efflux rate in the absence of the test compound. Compounds that are LAT1 substrates should increase the efflux rate of the radiolabeled amino acid compared with its efflux rate in the absence of test compound. We selected [^{3}H]-gabapentin as a probe substrate due to its selectivity for LAT1 relative to other membrane transporters.[8]

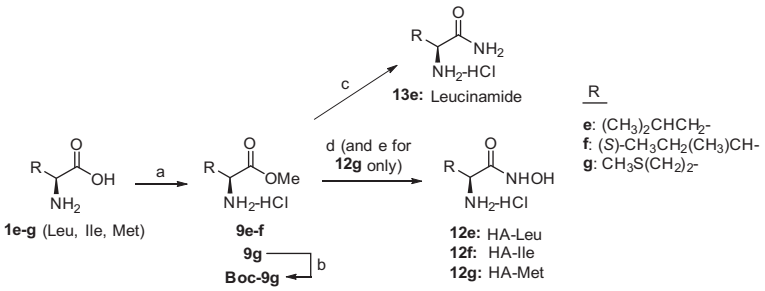
Acyl sulfonamides **10a**–**b** were prepared from protected amino acids according to methodology described by Drummond.[54] Tetrazole bioisostere **11** was synthesized from the primary amide of Cbz-protected Phe in 3 steps using a previously published route,[55] and our resulting NMR characterization was consistent with what had previously been reported. After discovering a lack of activity for tetrazole **11**, we choose not to pursue additional amino acid analogs. Hydroxamic acids (HA's) were synthesized using two different routes. Aromatic analogs (i.e., **12a**–**12d**) were prepared using methodology previously described by Ahlford and Adolfsson.[56] Due to problems with over-reduction of hydroxamic acids to give primary amide (e.g., **13e**) during hydrogenolysis of benzyl protected hydroxamic acid, we used a different route to prepare HA's of aliphatic amino acids (Scheme 1).

Hydroxamic acids of aliphatic amino acids Leu, Ile and Met (**12e**–**g**) were synthesized according to Scheme 1. Nucleophilic acyl substitution with hydroxylamine on methyl esters **9** gave low yields, but avoided having to use an amine protecting group for Leu and Ile analogs. However, reaction of **9g** with hydroxylamine to form HA-Met gave a complex mixture that could not be purified by recrystallization. We found that Boc protected **9g** gave a cleaner conversion to HA-Met, albeit the recrystallized yield was still relatively poor (20% for steps **d**–**e**). Since our objective was to obtain HA's of high purity with negligible levels of parent amino acids to avert a false positive result in our cell assay, we were generally unconcerned about isolated yield and the potential losses resulting from multiple recrystallization steps. Moreover, potentially better methods[57–60] for preparing HA's were not pursued, as the current routes provided satisfactory amounts of material for testing in a relatively short time period. In contrast, the yield for substitution with ammonia to generate leucinamide **13e** was significantly

Table 1
Exchange efflux rate and uptake inhibition of [^{3}H]-gabapentin in HEK-hLAT1 cells for carboxylic acid bioisosteres and their parent amino acids tested at 200 μM

Compound[a]	A	R	Efflux rate[b]	% inhibition[c]	pK_{a1}, pK_{a2}[d]
1a (Phe)	–CO_2H	$PhCH_2$–	3.6 ± 0.7	85 ± 0.6	1.8, 9.1
1b (Tyr)	–CO_2H	*p*-$HOPhCH_2$–	2.6 ± 0.4	68 ± 0.5	—
1h (Gly)	–CO_2H	H	0.78 ± 0.2	33 ± 3	—
1i (Arg)	–CO_2H	$NH_2(NH)CNH(CH_2)_3$–	0.75 ± 0.1	49 ± 2	—
10a	–$CONHSO_2Me$	$PhCH_2$–	0.61 ± 0.06	15 ± 0.7	1.8, 8.4
10b	–$CONHSO_2Me$	*p*-$HOPhCH_2$–	0.63 ± 0.03	12 ± 0.4	—
11		$PhCH_2$–	0.61 ± 0.01	11 ± 1	2.5, 7.8
12a (HA-Phe)	–CONHOH	$PhCH_2$–	1.3 ± 0.1	24 ± 0.5	6.9, 8.0
12b (HA-Tyr)	–CONHOH	*p*-$HOPhCH_2$–	1.0 ± 0.1	39 ± 30	—

[a] Cell assay data was obtained at least in triplicate. Amino acids and their corresponding derivatives possess *S* stereochemistry at the α-carbon.
[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.
[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.
[d] pK_a values for Phe **1a** were taken from Berg,[62] and the pK_a values for **10a**, **11**, and **12a** were measured at Analiza using a capillary electrophoresis technique.[61]



Scheme 1. Synthesis of compounds **13e** and **12e–12g**. Reagents and conditions: (a) $SOCl_2$, MeOH, **9e**: 56%, **9f**: 86%, **9g**: 67%; (b) Boc_2O, DCM, Boc-**9g**: 85%; (c) 7 N NH_3 in MeOH, 50 °C, sealed tube, **13e**: 60%; (d) 50% NH_2OH in water, MeOH or 1,4-dioxane, **12e**: 23%, **12f**: 3%, **12g**: 20% (2 steps); (e) 4 N HCl in 1,4-dioxane. **13e** and **12e–g** were purified by conversion to their HCl salts and recrystallization to >99% purity by HPLC.

better (60% recrystallized yield) than for the corresponding HA analogs. Generally HA's demonstrated poor solubility in both water and organic solvents; however, we found that solubility was dramatically improved by conversion to the hydrochloride salt.

Of these three bioisosteres, only the hydroxamic acid **12a** had significant activity in our *trans*-stimulation assay relative to non-substrates Gly and Arg (Table 1). We were surprised by this result. We had expected the tetrazole **11** and acylsulfonamides **10a** to have been better surrogates for the acidic carboxylic acid than **12a** given that the measured pK_{a1} values[61] for the former (pK_{a1} = 2.5 and 1.8, respectively) were much closer to that of parent amino acid Phe **1a** (pK_{a1} = 1.8) than HA-Phe **12a** (pK_{a1} = 6.9) was. It is worth noting that our pK_{a1} values were considerably lower than those reported for these bioisosteres when they were present as isolated functional groups,[34] which demonstrates as might be expected that the α-amino group depresses their pK_a as it would for an adjacent carboxylic acid.

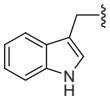
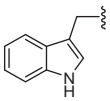
To determine whether HA's of other LAT1 amino acid substrates (e.g., Leu, Ile, Met) were LAT1 ligands, compounds of Table 2 were prepared and tested. All of the HA's, with the exception of **12h**, had diminished activity in both our *trans*-stimulation and *cis*-inhibition assays relative to the parent amino acids. Based on their % inhibition of [^{3}H]-gabapentin cell uptake or IC_{50} values, it is clear that HA's are weaker ligands of LAT1 than the parent amino acids. And none of the HA's had IC_{50} values below 200 μM in our assay. Conversely, all of the HA's demonstrated greater efflux rates of [^{3}H]-gabapentin from pre-loaded HEK-hLAT1 cells than did the negative controls Arg and Gly. The notable exception to this trend was HA-Gly **12h**, which we did not expect to be a LAT1 substrate by analogy to its non-substrate, parent amino acid Gly. Of the HA's tested, **12a** and **12e–g** (HA's of Phe, Leu, Ile, Met) demonstrated significant activity relative to the negative controls. The larger efflux rates measured for HA-Leu **12e** and HA-Ile **12f** relative to HA-Phe **12a** (1.5 vs 1.3 fmol/min) were juxtaposed with the activity of the parent amino acids, in which Phe **1a** demonstrated a superior efflux rate (3.6 fmol/min). It has previously been shown that both Leu and Ile have slightly greater LAT1 transport capacity (V_{max}) values than Phe.[63,64] Since the *trans*-stimulation assay relies upon the kinetics of exchange between intracellular [^{3}H]-gabapentin and extracellular test compound, it is conceivable that a similar trend for V_{max} applies to the HA's as it does to the parent amino acids. However, considering the bounce in our assay relative to the observed efflux rates, we cannot confidently distinguish the substrate activity of the HA's from each other.

Given the disparity in the literature[4,32,37] as to whether esters are LAT1 substrates, we also tested the Leu methyl ester **9e**, which was an intermediate in the preparation of HA-Leu **12e** (Scheme 1). And to further probe the SAR for close-in derivatives of the HA's (Table 2), we also decided to test the structurally-related primary amide Leucinamide **13e**. Though ester **9e** was recently reported to be a LAT1 substrate,[32] to our knowledge this was the first time that **13e** or any amino acid primary amide has been tested for LAT1 activity. Interestingly, **13e** did not demonstrate significant substrate activity (efflux rate: 0.69 fmol/min). This result also indicated that **13e** was sufficiently stable to the assay conditions so as not to generate adequate parent Leu **1e** to cause *trans*-stimulation. Furthermore, it is apparent that the hydroxamic acid '–OH' group plays an important role in the observed LAT1 activity; whether that be due to its effect on acidity, hydrogen bonding, or some other factor is currently unclear.

A different story unfolded for ester **9e**. In a *trans*-stimulation experiment performed by Nagamori,[32] both ester **9e** and its parent Leu appeared to have almost identical activity. In our hands, **9e** exhibited significantly less activity than parent Leu in both *trans*-stimulation (efflux rate: 2.1 vs 3.2 fmol/min) and *cis*-inhibition assays (IC_{50}: >200 μM vs 87 μM). One possible explanation for this disparity may be due to the fact that the cells used by Nagamori were different from the cells we used. Nagamori et al. used non-transfected HeLa S3 cells (a cervical cancer cell line) whereas, we used HEK-hLAT1 cells[52] that demonstrated 8-fold higher uptake of [^{3}H]-gabapentin relative to the control cell line HEK-EV (Supplementary material). LAT1 expression may have been higher in our transfected cells and the contribution of other transporters could differ between the two cell lines. We selected HEK cells due to their having relatively low levels of transporters,[65] so we would expect the observed activity in our assays to be due solely to LAT1. Our IC_{50} value for **9e** was consistent with earlier SAR presented by Uchino[4] that the methyl ester of phenylalanine poorly inhibited uptake of L-[^{14}C]-Phe into oocytes expressing LAT1. But the ostensible interpretation of the results from our *trans*-stimulation assay is the same as Nagamori's—that methyl ester **9e** does appear to be a LAT1 substrate, despite lacking an acidic carboxylic acid functional group.

Because of our concerns and those raised by others about the potential for a false positive result in LAT1 cell assays,[37] we evaluated how much parent Leu **1e** would need to be present as an impurity in test compounds (i.e., **9e**, **12e**, or **13e**), either from the synthesis or formed under the conditions of the cell assay, to result in a significant efflux rate (>1 fmol/min) in our *trans*-stimulation assay. We tested the efflux rate at concentrations ranging from 4 μM up to 200 μM covering a range of Leu **1e** impurity from 2% to 100%, respectively (in relation to previous studies). The background efflux rate (0.7 ± 0.05 fmol/min) was subtracted from total efflux and the net contribution to [^{3}H]-gabapentin efflux rate is depicted in Figure 1. The effect of increasing concentrations of Leu **1e** on the net LAT1 efflux rate was fitted to the Michaelis–Menten equation (K_m of 36.8 ± 9.8 μM and V_{max} of 1.99 ± 0.19 fmol/min). The K_m and V_{max} were similar to previously reported values.[64] A Leu **1e** concentration of 4 μM did not increase the LAT1 exchange rate significantly in comparison to background signal

Table 2
Exchange efflux rate and uptake inhibition of [^{3}H]-gabapentin in HEK-hLAT1 cells for hydroxamic acids, related carboxylic acid derivatives, and their parent amino acids

Compound[a]	X	R	Efflux rate[b]	% inhibition[c]	IC_{50} (μM)[d]
1a (Phe)	–OH	$PhCH_2$–	3.6 ± 0.7	85 ± 0.6	—
1b (Tyr)		*p*-$HOPhCH_2$–	2.6 ± 0.4	68 ± 0.5	—
1d (Trp)			1.6 ± 0.3	79 ± 0.6	—
1e (Leu)		$(CH_3)_2CHCH_2$–	3.2 ± 0.5	73 ± 0.7	87 ± 10
1f (Ile)		(*S*)-$CH_3CH_2(CH_3)CH$–	2.5 ± 0.1	—	150 ± 40
1g (Met)		$CH_3S(CH_2)_2$–	2.4 ± 0.1	—	180 ± 3
1h (Gly)		H	0.78 ± 0.2	33 ± 3	>200
1i (Arg)		$NH_2(NH)CNH(CH_2)_3$–	0.75 ± 0.1	49 ± 2	—
12a (HA-Phe)	–NHOH	$PhCH_2$–	1.3 ± 0.1	24 ± 0.5	—
12b (HA-Tyr)		*p*-$HOPhCH_2$–	1.0 ± 0.1	39 ± 30	—
12d (HA-Trp)			1.0 ± 0.1	35 ± 1	—
12e (HA-Leu)		$(CH_3)_2CHCH_2$–	1.5 ± 0.1	50 ± 1	>200
12f (HA-Ile)		(*S*)-$CH_3CH_2(CH_3)CH$–	1.5 ± 0.2	—	>200
12g (HA-Met)		$CH_3S(CH_2)_2$–	1.1 ± 0.01	—	>200
12h (HA-Gly)		H	0.70 ± 0.1	38 ± 1	>200
9e (Leu ester)	–OMe	$(CH_3)_2CHCH_2$–	2.1 ± 0.2	—	>200
13e (Leucinamide)	$-NH_2$		0.69 ± 0.01	—	>200

[a] Cell assay data was obtained at least in triplicate. Amino acids and their corresponding derivatives possess *S* stereochemistry at the α-carbon.
[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.
[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.
[d] For IC_{50} determinations, varying concentrations of each compound were added, from 0.1 μM to 500 μM. %[^{3}H]-gabapentin uptake at each concentration was normalized relative to % inhibition by BCH[66,67] at 2 mM, which was set to 100% inhibition.

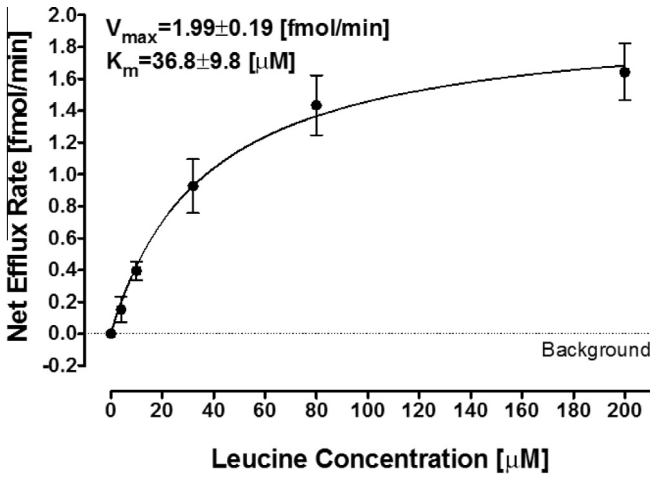


Figure 1. Plot of net exchange efflux rate (fmol/min) of [^{3}H]-gabapentin from pre-loaded HEK-hLAT1 cells versus Leu (**1e**) concentration (μM). Net efflux rate was calculated by subtracting the exchange rate without Leu (**1e**) (marked by a dotted line) from the efflux rate at Leu (**1e**) concentrations of 4, 10, 32, 80 and 200 μM. Solid line represents a non-linear regression fit of the data to Michaelis–Menten kinetics.

(*p* value = 0.146), and higher concentrations were required to facilitate the exchange of [^{3}H]-gabapentin. In fact, more than 10% of a parent Leu **1e** impurity would be required to fully account for HA-Leu **12e**'s LAT1 activation (1.5 ± 0.1 fmol/min). Nevertheless, contamination of test compounds with parent amino acid may result in increased efflux ratio, and this should be examined carefully when performing *trans*-stimulation assays.

Compelled by the implications of Figure 1, we carefully scrutinized the purity of all of our HA's by NMR and HPLC (Supplementary material), in particular checking for the presence of residual parent amino acid. We recrystallized all of the HA's at least once to improve purity, and the amount of parent amino acid detected by HPLC was less than 0.5%, and in most cases it was below our limit of detection. Thus, we conclude that for the HA's of Table 2 substrate activity was not due to parent amino acid carried over from the synthesis.

In addition to being vigilant about purity, we also performed a series of simple stability experiments. Thus, we exposed ^{13}C-labeled **9e** and **12e** (Fig. 2; synthesized using similar methods as described for unlabeled **9e** and **12e**, above) to conditions to mimic our cell assays, including incubation of compounds with 'buffer only' or with 'buffer and cells' for various periods of time. The resulting mixture was analyzed by ^{13}C NMR (Supplementary material).

Within the time period of our cell assay (~5 min), ^{13}C-**9e** hydrolyzed to give 4% parent amino acid, whereas ^{13}C-**12e** only gave a marginal increase in parent ^{13}C-**1e** relative to its initial amount (0.8% vs 0.4%, respectively) that was likely within the variability of NMR peak integration. To take the experiment further, we incubated compounds with cells for an hour at 37 °C. This resulted in a moderate increase in the amount of ^{13}C-**1e** (12%) from ester ^{13}C-**9e**, but only a nominal amount (1%) arising from HA-Leu ^{13}C-**12e** hydrolysis. Even after incubation of HA-Leu ^{13}C-**12e** with cells for 5 h at 37 °C, only 3% ^{13}C-**1e** was observed in the ^{13}C NMR spectrum.

Due to ^{13}C NMR analysis requirements, these stability experiments had to be performed with 1 mg of compound/well, which was ~25–50× more compound than typically used in our cell assays, done at 200 μM concentration. Consequently, we can't rule

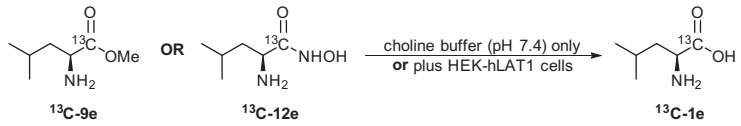


Figure 2. Carbon-13 labeled leucine derivatives [^{13}C]-**9e** (methyl ester) and [^{13}C]-**12e** (HA-Leu) used in stability experiments to assess the amount of parent leucine that could form under the conditions of the LAT1 cell assays.

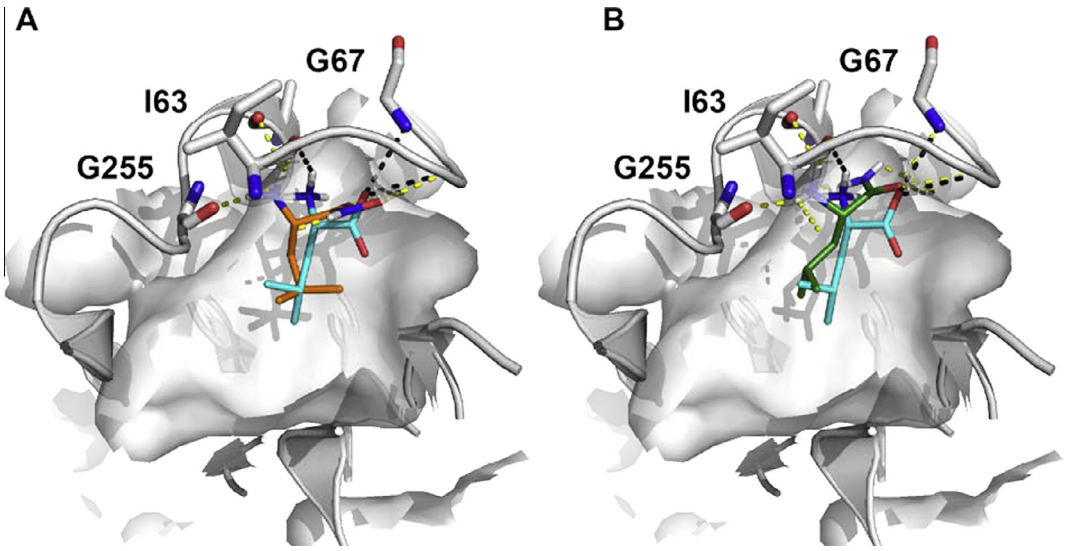


Figure 3. Predicted binding mode of LAT1 with leucine and its analogs. Predicted pose of the known substrate leucine **1e** is shown in cyan sticks, and the two leucine analogs including (A) the recently discovered substrate HA-Leu **12e** and (B) non-ligand Leucinamide **13e** are illustrated with orange and green sticks, respectively. The hydrogen bonds between LAT1 binding site residues and the leucine analogs are shown as dashed lines, including **1e** (yellow), **12e** (black), and **13e** (black).

out the possibility of enzyme saturation at this higher concentration, and that either **9e** and/or **12e** might be undergoing intracellular enzymatic cleavage as the assay is normally conducted. However, it was previously shown[68] that leucinamide **13e** is more sensitive to enzymatic hydrolysis than the leucine methyl ester **12e**. So, if enzyme-catalyzed formation of parent Leu was problematic, it seems likely that leucinamide would also have given a false positive; yet, **13e** lacked activity in our *trans*-stimulation assay.

Upon relating these stability results to Figure 1, it appears that the concentration of parent Leu **1e** within the timeframe of our cell assay from either methyl ester **9e** or HA-Leu **12e** (~8 and 2 μM, respectively) does not explain our *trans*-stimulation assay results (2.1 and 1.5 fmol/min, respectively). For the observed activity to be solely due to parent amino acid, Figure 1 suggests that Leu would have to be present at ~80 and ~30 μM concentrations for **9e** and **12e**, respectively, and all within the 3 min of the assay. Our stability experiments indicated that ⩽10% of these levels were actually present, supporting the notion that the observed exchange efflux of [^{3}H]-gabapentin (Table 2) was mostly caused by the test compounds themselves. Even so, it is probable that a small fraction of the activity was due to Leu, particularly for the less stable methyl ester **9e**.

Though the atomic structure of the human LAT1 is not known, we have developed a homology model based on a structure of a related transporter, the arginine-agmatine transporter AdiC from *Escherichia coli*.[51,69] This model has been recently refined using newly characterized ligands and improved LAT1/AdiC alignment.[40] The LAT1 model helped rationalize the amino acid selectivity among amino acid transporters, and virtual screening against this model followed by experimental testing identified previously unknown LAT1 ligands.[51] Docking of **12e** against our LAT1 model[40] suggests that hydroxamic acids establish hydrogen bonds with backbone atoms of Ile63 and Gly67 in a manner similar to that of LAT1 amino acid ligands such as leucine (Fig. 3A). We postulate that maintaining these hydrogen bonds is important for activity, and that this is the primary reason hydroxamic acids are LAT1 substrates. Interestingly, docking of non-ligand leucinamide **13e** (Fig. 3B) using two different docking programs (i.e., FRED[70] and Glide SP;[71] Supplementary material) does not rule out that **13e** is a LAT1 ligand. We therefore estimated the binding energies of Leu (**1e**), Leu methyl ester (**9e**), HA-Leu (**12e**), and leucinamide (**13e**) to LAT1 using Molecular Mechanism Generalized Born Surface Area (MMGBSA) calculated by Prime (Schrödinger suite).[72] Though the predicted ΔG_{bind} values of −79, −64, and −58 kcal/mol for **1e**, **12e**, and **13e**, respectively, correlated with experimental data, the predicted ΔG_{bind} for **9e** (−52 kcal/mol) did not. As a large component of the calculated binding energies was due to electrostatic interactions (i.e., $\Delta G_{Coulomb}$ of −63, −53 and −47 kcal/mol for **1e**, **12e** and **13e**, respectively), it is possible that our calculations are underestimating other interactions (e.g., dipole–dipole) with LAT1 that exist for ester **9e** which account for its observed activity.

Based on our results and those recently reported by Nagamori,[32] it appears that the previous view that LAT1 substrates *must possess* an acidic functional group needs to be revised. As most of the earlier conclusions[4,37,73] were based on inhibition experiments (e.g., *cis*-inhibition or rat brain perfusion) at fixed concentrations of test compound, it is possible that those assays were not sensitive enough to detect substrates such as esters with weaker interactions with LAT1. The *trans*-stimulation assay may be more sensitive to identify weak ligands, as it is based on the exchange of pre-loaded substrates (e.g., [^{3}H]-gabapentin) only for test compounds that employ LAT1 to cross a cell membrane, rather than inhibition potency.

Though we do not currently have an explanation for why tetrazoles and acylsulfonamides lacked activity, our data point toward LAT1 binding being less sensitive to the pK_a of the carboxylic acid surrogate and more sensitive to its H-bonding capabilities. Thus, our results support the observation made by Nagamori[32] that both oxygens of an amino acid carboxylic acid are likely involved in H-bonding with LAT1. We are currently expanding our work to include additional carboxylic acid bioisosteres[35] to test this hypothesis.

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Supplementary data

Supplementary data (full experimental details, compound characterization data, IC_{50} curves, stability data and model refinement and ligand docking description) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.09.001.

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