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Structural properties for selective and efficient L-type amino acid transporter 1 (LAT1) mediated cellular uptake

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School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, P.O. Box 1627, FI-70211, Kuopio, Finland

Corresponding author

Jussi Kärkkäinen; Phone: +358 50 499 3955; E-mail: jussi.karkkainen@uef.fi

Abbreviations

ARPE-19, a human retinal pigment epithelial cell line; ATCC, American Type Culture Collection; ASCT2, alanine-serine-cysteine transporter 2; BBB, Blood Brain Barrier; BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; CNS, Central Nervous System; CYP3A4, cytochrome P450 3A4; DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal Bovine Serum; HBSS, Hank's balanced salt solution; HEK cells, human embryonic kidney cells; HeLa S3 cells, human derived cervix adenocarcinoma cells; LAT1, L-Type Amino Acid Transporter 1; LAT2, L-type Amino Acid Transporter 2; LC-MS/MS, liquid chromatography tandem mass spectrometry; MCF-7, human breast adenocarcinoma cell line; MDCK cells, Madin-Darby canine kidney cells; MDR1, multidrug resistance protein 1; mTOR, mechanistic/mammalian target of rapamycin; OCT2, organic cation transporter 2; PMAT, Plasma Membrane Monoamine Transporter; 3D-QSAR, 3-Dimensional Quantitative Structure Activity relationship model

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Abstract

L-type amino acid transporter 1 (LAT1) is a sodium-independent exchanger transporting large neural amino acids and several amino-acid mimicking drugs across the cell membranes. LAT1 is highly expressed at the blood brain barrier (BBB) and in numerous cancer cells and is therefore a potential drug target. However, structural features affecting the ability to bind to LAT1 and the cellular translocation by LAT1 are unclear. In the present study we determined the binding to and transport through human LAT1 of several compounds into the human breast adenocarcinoma cells (MCF-7). We show that the meta-conjugation of L-phenylalanine increases binding to human LAT1 compared to para-conjugation or aliphatic amino acid moiety. Furthermore, large, rigid and aromatic meta-substituted L-phenylalanine derivative enabled selective and efficient LAT1-mediated cellular uptake. Our results also demonstrates that in addition to binding studies, it is of utmost importance to determine the cellular accumulation of compounds. It provides crucial information on transport efficiency and selectivity of transport mechanisms that the compounds are able to utilize. Overall, these structural findings and the methodology used herein are exploitable to design LAT1-utilizing compounds, such as markers for cancer imaging and drug molecules, enabling more effective and safer treatments for cancer in the future.

Keywords

L-type amino acid transporter 1 (LAT1), targeted drug delivery, transporter-mediated drug delivery, transporter substrate, cell uptake

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1. Introduction

L-type amino acid transporter 1 (LAT1) belongs to the L-type amino acid transporter family and is a sodium-independent, heterodimeric transmembrane exchanger (Kanai, et al. 1998; Yanagida, et al. 2001). LAT1 transports across the cell membranes large neutral amino acids such as L-leucine, L-phenylalanine and L-tryptophan, as well as several amino acid-mimicking drugs including levodopa, gabapentin and melphalan (Cornford, et al. 1992; Dickens, et al. 2013; Kageyama, et al. 2000; Kanai, et al. 1998; Yanagida, et al. 2001). LAT1 is found mostly in the brain, testis and placenta (Boado, et al. 1999; Kanai, et al. 1998; Yanagida, et al. 2001). More specifically, LAT1 is highly expressed at the blood brain barrier (BBB). LAT1 is also overexpressed in various cancer cells, including breast cancer, non-small cell lung cancer and glioblastoma multiforme (Furuya, et al. 2012; Kaira, et al. 2008a; Kobayashi, et al. 2008). The functional level of LAT1 correlates with cancer progression; metastases and high grade tumors express greater amounts of LAT1 (Furuya, et al. 2012; Kaira, et al. 2008b; Papin-Michault, et al. 2016). Moreover, cancer cell proliferation decreases when the function of the LAT1 is inhibited (Huttunen, et al. 2016b; Oda, et al. 2010; Shennan and Thomson 2008). Therefore, LAT1 is an interesting drug target: it can be utilized as a diagnostic marker for cancer imaging; as a therapeutic target to starve cancer cells by inhibiting its function; or as a delivery mechanism for carrier-mediated transport of drugs to cancer cells or through the BBB to the central nervous system (CNS) (Jin, et al. 2015; Rautio, et al. 2013).

A molecule utilizing LAT1 may mimic a natural substrate of LAT1, as do the small size compounds gabapentin and levodopa, or constitute a part of larger molecule containing structural features for LAT1 recognition (Gynther, et al. 2010; Gynther, et al. 2016; Huttunen, et al. 2016b; Peura, et al. 2011; Peura, et al. 2013; Puris, et al. 2017). Several prodrugs of such type with various amino acid promoieties have been demonstrated to utilize

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LAT1 for their BBB-permeation in *in situ* rat brain perfusion experiments or for cellular accumulation in *in vitro* studies, or for both. However, while structural properties responsible for efficient LAT1 affinity have been successfully characterized (Ylikangas, et al. 2013; Ylikangas, et al. 2014), properties affecting the binding to LAT1 and the cellular translocation by LAT1 are not comprehensively understood. In addition, amino acid derivatives binding to LAT1 may also be substrates for other amino acid transporters, such as monocarboxylate transporters 8 and 10 (MCT8 and MCT10) (Kinne, et al. 2011) or organic anions transporting polypeptides (OATPs) (Huttunen, et al. 2016b), which can complicate targeted drug delivery via LAT1, and e.g. impair the selective delivery into the cancer cells.

In this study, our aim was to characterize systematically the structural features for efficient and selective binding to and transport through human LAT1. Knowlegde of these features enables more rational design of LAT1-targeted compounds through better understanding of structure-activity relationship of a transporter and a ligand/substrate. The efficient LAT1-mediated disposition and high selectivity of compounds for LAT1 over other amino acid transporters is of great importance for the success of the transporter mediated drug targeting approach. In order to achieve our aim we evaluated the ability of ten selected compounds to bind to human LAT1 (Table 1) in a LAT1-expressing cell line (human breast adenocarcinoma cancer cell line; MCF-7). Furthermore, we studied LAT1-selective translocation and uptake of compounds into these cells. The selected compounds included clinically used LAT1-utilizing drugs, investigational amino acid derivatives and natural LAT1 substrates. LAT1-selectivity was determined over two other amino acid transporters, L-type amino acid transporter 2 (LAT2) and alanine-serine-cysteine transporter 2 (ASCT2). ASCT2 was selected for the selectivity studies because this transporter is overexpressed in cancerous cells (Fuchs and Bode 2005; Wise and Thompson 2010) sharing mutual ligands with LAT1 (Colas, et al. 2015). LAT2 was selected beacause it has overlapping substrate

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specificity with LAT1, but it exists ubiquitously in normal tissues (Pineda, et al., 1999; Rossier, et al., 1999; Segawa, et al., 1999). The results of this study contribute to the current knowledge of efficient and selective LAT1-mediated uptake of compounds, facilitating more rational design of compounds utilizing LAT1 to reach CNS or selectively to target cancer cells overexpressing LAT1.

2. Materials and methods

2.1. Synthesis of amino acid derivatives

Synthesis of compound **7** (L-5-methyltryptophan methyl ester (methyl (S)-2-amino-3-(5-fluoro-1H-indol-3-yl)propanoate)) was performed with reagents obtained from Sigma-Aldrich (St. Louis, MO, USA), Acros Organics (Waltham, MA, USA) or Merck (Darmstadt, Germany). Reaction was monitored by thin-layer chromatography using aluminum sheets coated with silica gel 60 F_{245} (0.24 mm) with suitable visualization. Purification by flash chromatography was performed on silica gel 60 (0.063-0.200 mm mesh), eluting with CH_2Cl_2/MeOH solution. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 spectrometer (Bruker Biospin, Fällanden, Switzerland) operating at 500.13 MHz and 125.75, respectively, using tetramethylsilane as an internal standard. ESI-MS spectra were recorded by a Finnigan LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray ionization source. Purity of over 95% was confirmed by elemental analysis with a Perkin Elmer 2400 Series II CHNS/O organic elemental analyzer (Perkin Elmer Inc., Waltham, MA, USA).

The compound **7** was prepared according previously published procedures (Tang, et al. 2008) to yield off white solid, 23 mg (97%). ^{1}H NMR ($(CD_3)_2SO$): δ ppm 11.15 (s, 1 H), 8.28-8.08 (bs, 2H), 7.37 (dd, *J* = 8.8, 4.5 Hz, 1H), 7.29 (d, *J* = 2.5 Hz, 1H), 7.27 (dd, *J* = 10.2, 2.5 Hz, 1H), 6.94 (dt, *J* = 9.1, 2.5 Hz, 1H), 4.27 (t, *J* = 6.3 Hz, 1H), 3.69 (s, 3H), 3.21 (d, *J* = 6.3 Hz,

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2H). ^{13}C NMR ($(CD_3)_2SO$): δ ppm 169.93, 155.95, 132.86, 127.18 (d), 126.97, 112.49 (d), 109.25 (d), 106.76 (d), 102.72 (d), 52.70, 52.66, 26.18. MS (ESI^+) for $C_{12}H_{14}FN_2O_2$ $(M+H)^+$: Calcd 237.26, Found 237.01. Elemental analysis (C12H13FN2O2), C, H, N.

The characterization and purity for synthetic ketoprofen (**4**, **5** and **6**) and valproic acid (**1, 2, 3**) derivatives have been described in our previous publications (Gynther, et al. 2010; Puris, et al. 2017; Ylikangas, et al. 2014)

2.2. Other chemicals

All solvents and reagents used for *in vitro* –studies and for analytics were high purity of analytical or HPLC grade. Solvents and reagents were purchased from J.T. Baker (Denventer, The Netherlands), Riedel-de Haën (Seelze, Germany), Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany). The water used in the studies was purified (Milli-Q Gradient system, Millipore, Milford, MA, USA).

2.3. Selection of compounds

We selected compounds **1-6** (Table 1) based on the 3-Dimensional Quantitative Structure Activity (3D-QSAR) model of rat LAT1 binding site (Ylikangas, et al. 2014). Compounds **1**, **2** and **3** are valproic acid derivatives of L-phenylalanine. L-phenylalanine is substituted at the *meta* (**1, 2**) or *para* (**3**) position of aromatic ring and compound **1** includes a methylene group between the aromatic ring of amino acid and valproic acid. They all have at least moderate affinity to LAT1 (rat and human) and are transported through BBB (rat) and cell membrane (human) via LAT1 (Gynther, et al. 2016). Compounds **4** and **5** are ketoprofen derivatives of L-phenylalanine. L-phenylalanine is substituted at the *meta* (**4**) or *para* (**5**) position of aromatic ring. They both have high affinity to human LAT1 and are transported through the cell membrane in human retinal pigmented epithelial cells (ARPE-19) as well as through the BBB via LAT1 in mice (Puris, et al. 2017). Compound **6** is L-lysine derivative of ketoprofen,

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which can bind to and permeate through the BBB via rat LAT1 (Gynther, et al. 2010), but also through the cell membrane via human LAT1 (Puris, et al. 2017). Compounds **1**-**6** have amide bond between the valproic acid or ketoprofen and amino acid and they were tested to be stable under the studied conditions. Although they have been found to release their parent drug *in vivo* and thus behave as prodrugs, they all are extremely stable *in vitro*. Since bioconversion is not being studied here, we refer these compounds as stable LAT1-derivatives. Compound **7** is a methyl ester of 5-fluoro-L-tryptophan and it is included to clarify the contradictory role of acidic functional group for LAT1-substrates (Rautio, et al. 2015; Zur, et al. 2016). While being stable during the *in vitro* studies in HBSS, compound **7** degraded completely when the cells were lysed in 0.1 M NaOH. Finally, the commercial LAT1-substrates melphalan (Cornford, et al. 1992) and gabapentin (Dickens, et al. 2013), as well as BCH, an inhibitor of system L-amino acid transporters (Kim, et al. 2008), were selected for the *in vitro* experiments.

2.4. Cell line and culture conditions

MCF-7 human breast adenocarcinoma cells (HTB-22) were purchased from the American Type Culture Collection (ATCC). MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine (2 mM), fetal bovine serum (FBS) (10%), penicillin (50 IU/mL) and streptomycin (50 µg/mL) at 37 °C in a humidified incubator with 5 % CO_2. All *in vitro* -experiments were performed while the passage number of the cells was in the range of 9 to 38.

2.5. Expression and function of LAT1, LAT2 and ASCT2 in MCF-7 cells

The expression and function of LAT1 and LAT2 was detected as previously described (Huttunen, et al. 2016a), with minor modifications. For ASCT2 and 4F2hc the primer probe mixes used were Hs01056542_m1 (ASCT2, SLC1A5) and Hs00374243_m1 (4F2hc). The

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function of LAT1, LAT2 and ASCT2 was determined by studying the kinetics of cellular uptake of known radiolabeled substrates [^{14}C]-L-leucine (LAT1), [^{14}C]-L-alanine (LAT2) and [^{3}H]-L-glutamine (ASCT2). Different amounts of unlabeled L-leucine, L-alanine or L-glutamine were added to the uptake buffer (Na^+ -free HBSS for LAT1 and LAT2 studies, and HBSS with Na^+ for ASCT2 studies) to give increasing total ([^{14}C]/[^{3}H] plus unlabeled L-leucine, L-alanine or L-glutamine) substrate concentrations ranging from 0.16 to 1000 µM (L-leucine and L-alanine) and from 15.6 to 4000 µM (L-glutamine). The uptake time was 5 min (kinetic studies) or 0.5 – 30 min (time course study of 0.16 µM [^{14}C]-L-leucine). Furthermore, the uptake of substrates was studied in the presence and absence of Na^+ to determine LAT2-mediated L-alanine transport and to confirm that L-leucine was not transported via Na^+-dependent transporters and in contrary, that L-glutamine was transported via Na^+-dependent transporter. The functionality of LAT1, LAT2 and ASCT2 in MCF-7 cells was monitored as described above during the *in vitro* tests.

2.6. Determination of the LAT1, LAT2 and ASCT2 binding

The ability of compounds to bind to LAT1, LAT2 and ASCT2 was studied as described previously (Rautio, et al. 2015). Briefly, after pre-incubation the cells were incubated for 5 min at room temperature in 250 µL of uptake medium, containing 0.157 µM of a known LAT1 substrate [^{14}C]-L-leucine (PerkinElmer, Boston, MA, USA), 10 µM of a known LAT2 substrate [^{14}C]-L-alanine (PerkinElmer, Boston, MA, USA) or 0.05 µM of a known ASCT2 substrate [^{3}H]-L-glutamine (PerkinElmer, Boston, MA, USA) in the presence or absence of compounds (at a compound concentration of 50 µM with [^{14}C]-L-leucine and [^{14}C]-L-alanine, 100 µM with [^{3}H]-L-glutamine) (n = 3 of each experiment). Unlabeled L-leucine, L-alanine or L-glutamine at 1 mM (L-leucine and L-alanine) or at 2 mM (L-glutamine) concentration served as positive controls. In ASCT2-affinity studies, HBSS with sodium was used at all stages and 500 µM L-leucine (Sigma-Aldrich, St. Louis, MO, USA) was added to test

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solution to rule out the exchange mechanism of LAT1. Subsequently, the cells were washed three times with ice-cold HBSS on top of an ice-bath. The cells were then lysed with 500 µL of 0.1 M NaOH and the lysate was mixed with 3.5 mL of Emulsifier safe cocktail (PerkinElmer, Waltham, MA, USA). The radioactivity was measured by liquid scintillation counting (Wallac 1450 MicroBeta; Wallac Oy, Finland).

2.7. Cellular uptake of compounds

Cellular uptake of compounds was studied over the concentration range of 6.25-200 µM (6.25, 12.5, 25, 50, 100 and 200 µM concentrations). After pre-incubation the cells were incubated for 10 min at room temperature in 250 µL of HBSS buffer containing different concentrations of compounds. Uptake was terminated by removing the test solutions and washing the cells three times with 500 µL of ice-cold HBSS on top of an ice-bath. The cells were lysed with 250 µL of 0.1 M NaOH. The concentrations of compounds (n = 3 of each concentration) in supernatants were determined by quantitative liquid chromatography with triple quadrupole mass spectrometric detection. The concentration was calculated from the standard curve that was prepared by spiking known amounts of each compound to the cell lysate. Results were normalized per well of protein content as measured by a protein assay kit (Bio-Rad Laboratories, Germany), using bovine serum albumin (Sigma-Aldrich, USA) as the standard. The protein concentrations on each plate were determined as the mean of three samples. To reveal LAT2- and ASCT2-mediated cellular uptake, the uptake of compounds was studied as described above in the presence of 2 mM L-alanine (Sigma-Aldrich, St. Louis, MO, USA) (LAT2 inhibition) or Na^+-free HBSS as a uptake buffer (ASCT2 inhibition). Cellular uptake of compounds was also studied with 100 µM of previously developed LAT1-inhibitor (Huttunen, et al. 2016a) as described above, with the following modifications: the cells were pre-incubated for 10 min in 500 µL of HBSS buffer or HBSS containing 100 µM LAT1-inhibitor; after pre-incubation, the cells were incubated for 10 min in 250 µL of HBSS

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containing 10 µM of investigated compound in the presence or absence of 100 µM LAT1-inhibitor; concentrations (pmol/mg/min) for uptake with and without LAT1-inhibitor were calculated from the standard curve and results are expressed as the percentage of the total cell uptake without inhibitor.

2.8. Sample preparation

An aliquot of 190 µL was taken from the lysates and 10 µL perchloric acid (10% v/v) was added for protein precipitation. Samples were vortexed, incubated on ice for 5 min and centrifuged for 10 min (14,000 rpm) at 4 °C. Then, 140 µL of supernatants were transferred to the 96-well collection plates (Captiva 96-well Collection Plates, Agilent Technologies) and 10 µL of internal standard, dissolved in methanol, was added. Finally, the plates were covered (Captiva pierceable 96-well collection plate cover, Agilent Technologies).

2.9. Analytical methods

Compounds were quantified from *in vitro* samples by liquid chromatography (Agilent 1200 series Rapid Resolution LC System, Agilent Technolgies, Waldbronn, Germany) coupled with and electrospray ionization (ESI) triple quadrupole mass spectrometer (Agilent 6410 Triple Quadrupole LC/MS, Agilent Technologies, Palo Alto, CA, USA). 5 µL of sample solution was injected onto a reversed phase HPLC column (Zorbax Eclipse XDB-C18 Rapid Resolution HT 4.6 x 50 mm, 1.8 µm, Agilent Technologies, Palo Alto, CA, USA). The column temperature was 40 °C, flow rate 0.3 mL/min (for compounds **1, 2, 3, 7,** melphalan, gabapentin, BCH and losartan) or 0.5 mL/min (for compounds **4, 5, 6** and diclofenac). Gradient elution was used for compounds **1, 2, 3, 7**, melphalan, gabapentin, BCH and losartan (0-4 min: 10 → 90 % B, 4-6 min: 90 % B, 6-6.1 min: 90 % → 10 % B, 6.1-9 min: 10% B) and for compounds **4, 5, 6** and diclofenac (0-1 min: 20 % → 80 % B, 1-4.5 min: 80 % B, 4.5-4.6 min: 80 % → 20 % B, 4.6-8 min: 20 % B) with water as eluent A containing 0.1

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% (v/v) of formic acid and acetonitrile as eluent B (for compounds **4, 5, 6, 7,** melphalan, gabapentin, BCH, losartan and diclofenac) or methanol as eluent B (for compounds **1, 2, 3** and losartan). The sample tray was maintained at 10 °C and the following ionization conditions were used: ESI positive ion mode; drying gas (nitrogen) temperature 300 °C; drying gas flow rate 8 L/min; nebulizer pressure 40 psi and capillary voltage 4000 V. Detection was performed using multiple reaction monitoring (MRM) with the following transitions: *m/z* 321 → 131.8 for compound **1**, *m/z* 307 → 117.9 for compound **2**, *m/z* 307 → 121.6 for compound **3**, *m/z* 416.8 → 134.9 for compounds **4** and **5**, *m/z* 383 → 84 for compound **6**, *m/z* 306 → 105 for melphalan, *m/z* 172 → 137 for gabapentin, *m/z* 156 → 110 for BCH, *m/z* 223 → 206 for compound **7**, *m/z* 423 → 207 for losartan (internal standard) and *m/z* 296.1 → 250 for diclofenac (internal standard). In analytics for compound **7** *m/z* 223 was monitored, since it was stable in HBSS, but decomposed immediately in 0.1 M NaOH from 236 to 222. The chemical stability was studied in HBSS and in 0.1 M NaOH and the samples were analyzed by LC-MS.

2.10. Statistical Analysis

Graphpad Prism v. 5.03 software (GraphPad Software, San Diego, CA, USA) was used for graphs and statistical analysis. Statistical differences between groups were tested using one-way ANOVA followed by Dunnett´s or Tukey`s multiple comparison test or Student's t-test (comparison of cell uptake with or without LAT1-inhibitor). Probability values of < 0.05 were considered statistically significant. The data are expressed as mean ± SEM (cellular accumulation data) or as mean ± SD (inhibition studies).

3. Results

3.1. Binding of compounds to LAT1, LAT2 and ASCT2

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Competitive inhibition assays were carried out in MCF-7 cell line to evaluate the ability of the selected compounds to bind to LAT1, LAT2 and ASCT2. The expression and function of LAT1, LAT2 and ASCT2 in MCF-7 cells were determined (Supplemental Fig. 1). Each studied compound was evaluated as a LAT1, LAT2 and ASCT2 ligand by determining their ability to inhibit the transport of [^{14}C]-L-leucine (LAT1 substrate), [^{14}C]-L-alanine (LAT2 substrate) or [^{3}H]-L-glutamine (ASCT2 substrate), at concentrations of 50 µM (inhibition of [^{14}C]-L-leucine and [^{14}C]-L-alanine) or 100 µM (inhibition of [^{3}H]-L-glutamine), respectively.

The inhibition of cellular uptake of [^{14}C]-L-leucine varied from 96% to ~15% (Fig. 1a). Compounds **1, 2, 4, 5** and **6** and melphalan resulted in the most significant inhibitions (94%, 84%, 96%, 88%, 78% and 61%, respectively) and gabapentin and compound **7** the lowest inhibitions (14% and 13%, respectively). *Meta*-substitution and rigidity of amino acid as well as size and rigidity of the substituent correlated with greater inhibition of [^{14}C]-L-leucine uptake (Table 1). The compounds with highest inhibition (**1, 2** and **4**) were the *meta*-derivatives of L-phenylalanine (84%-96%). The inhibition of [^{14}C]-L-leucine uptake decreased with *para*-conjugation of L-phenylalanine, and was evident especially in the comparison of valproic acid derivatives **2** and **3**, which resulted in 84% and 40% inhibitions, respectively. In the case of valroic acid derivatives, the LAT1-binding was not affected by the presence of a methylene group between the amide bond and aromatic ring of L-phenylalanine (94% inhibition by compound **1** vs. 84% inhibition without methylene group in compound **2**). Furthermore, the inhibition of [^{14}C]-L-leucine uptake decreased when using the more flexible non-aromatic L-lysine amino acid moiety; L-lysine-ketoprofen derivative **6** inhibited [^{14}C]-L-leucine uptake almost 20% units less than aromatic L-phenylalanine-ketoprofen derivative **4** (96% and 78% inhibitions, respectively). In addition, ketoprofen derivative **5** (88%) inhibited [^{14}C]-L-leucine uptake by almost 50% units more than corresponding valproic acid derivative **3** (40%). Also the *para*-substituted L-phenylalanine derivative melphalan showed 20% units

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stronger inhibition than the corresponding *para*-substituted valproic acid derivative **3**. BCH, a known inhibitor of L-type amino acid transporters (Kim, et al. 2008), exhibited modest inhibition (40%) and gabapentin and compound **7** displayed weak inhibitions (14% and 13%, respectively).

None of the compounds was able to inhibit cellular uptake of [^{14}C]-L-alanine (10 µM) or [^{3}H]-L-glutamine (0.05 µM) significantly (Fig. 1b and c). In contrast, *para*-substituted ketoprofen derivative **5** and L-lysine-ketoprofen derivative **6** increased accumulation of [^{14}C]-L-alanine into the MCF-7 cells (Fig. 1b). Similar trend was also observed for several other compounds, such as for compounds **2**, **3** and **7** as well as for melphalan and gabapentin, but for these the increase of [^{14}C]-L-alanine uptake was not statistically significant.

Therefore, according to these results, it can be concluded that all compounds were able to bind better to LAT1 than to LAT2 or ASCT2. Moreover, some of the compounds designed to bind to LAT1 (e.g. compounds **1** and **4**) had greater affinity for LAT1 compared to LAT1 inhibitor BCH and known LAT1 substrates melphalan and gabapentin.

3.3. Cellular uptake of compounds

Uptake of [^{14}C]-L-leucine into the MCF-7 cells increased with time and was linear up to 10 min (Supplemental Fig. 1a). Based on this result, LAT1-mediated cellular uptake of all compounds into the MCF-7 cell was studied at room temperature after 10 min incubation at 6.25 – 200 µM of compounds in Na^+ -free uptake buffer. In addition, cellular uptake of compounds was also studied with 100 µM of reported LAT1-inhibitor. To probe possible ASCT2-mediated transport, cellular uptake of compounds was studied in buffer with Na^+. Possible LAT2-mediated uptake was assessed in Na^+ -free buffer containing 2 mM L-alanine.

Presence of the previously developed LAT1-inhibitor (Huttunen, et al. 2016a) reduced total cell uptake of compounds **2** and **6** as well as of melphalan, gabapentin, and BCH significantly

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at a concentration of 100 µM (Fig. 3). The decline was largest for the small size compounds gabapentin and BCH, which exhibited in the [^{14}C]-L-leucine binding study only weak to modest inhibitions. In contrast, cell uptake of compounds **4**, **5**, and **7** remained equal despite the presence of LAT1-inhibitor and increased for compounds **1** and **3**. This may imply that the inhibitor is able to block the uptake of small compounds that are rapidly and extensively transported via LAT1 into the cells, while larger compounds with high binding affinity, such as compounds **1-6**, were able to efficiently compete with the inhibitor for LAT1 or use another transporter for their cell entry when LAT1 was inhibited. This can explain the equal or even increased uptake result.

All compounds exhibited concentration-dependent cellular uptake (Fig. 2). Total cellular uptake ranged from nanomole (nmol/mg protein/min) to picomole (pmol/mg protein/min) (compounds **1**-**6**) and small size compounds (gabapentin, BCH and compound **7**) had the largest absolute cellular uptake values (Fig. 2). LAT1-substrate gabapentin and transportable LAT1-inhibitor BCH were transported efficiently via LAT1 to the MCF-7 cells (Fig. 2). Cellular uptake of BCH followed typical Michaelis-Menten kinetic profile and V_{max} and K_m values were 1653 ± 111 pmol/mg protein/min and 81 ± 12 µM, respectively (Fig. 2). Also cellular uptake of compound **7** was efficient and saturable following typical Michaelis-Menten curve (Fig. 2). However, L-alanine was able to decrease the total uptake of compound **7**; V_{max} values with and without 2 mM L-alanine were 2070 ± 164 pmol/mg protein/min and 2664 ± 137 pmol/mg protein/min, respectively (Fig. 2). Thus, in addition to LAT1 also transporter(s) sensitive to L-alanine was involved in cellular uptake of compound **7**. Overall, cellular uptake of the larger compounds **1-6** and melphalan was less efficient than of the smaller compounds BCH, gabapentin and **7**.

The concentration-velocity profile of compounds **1-6**, melphalan and gabapentin did not exhibit the hyperbolic saturation curve during the 10 min uptake experiment at the

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concentration range used (Fig. 2). Instead of typical Michaelis-Menten curve, two-phase and non-typical/sigmoidal type of uptake profiles were observed. Therefore, for these compounds we characterized further the transport system and converted the cellular uptake data to Eadie-Hofstee plots (Fig. 2 insets).

For *para*-substituted L-phenylalanine derivative melphalan, kinetic analysis confirmed the biphasic profile (Fig.2 inset), which denotes the presence of two separate transporter mechanisms. Melphalan was transported via LAT1 at concentrations below 50 µM and other transporter(s) started to carry melphalan to the MCF-7 cells at higher concentrations. This other transport mechanism was sensitive to L-alanine; cellular uptake of melphalan was lower with than without extracellular L-alanine (Fig. 2). Similarly as for melphalan, kinetic analysis confirmed a biphasic profile and thus, presence of two separate transporter mechanisms, for the other aliphatic (valproic acid) L-phenylalanine derivatives **2** and **3** (Fig. 2 and insets). For compound **2** this second transport mechanism was not dependent on extracellular sodium or L-alanine. In turn, compound **3** was a substrate for sodium-dependent transporter; uptake of **3** was significantly higher with extracellular sodium than in a sodium-free environment. In contrast, aliphatic *meta*-substituted L-phenylalanine derivative with a methylene group between the amide bond and aromatic ring (compound **1**) was transported via LAT1 more efficiently and selectively to the cells than its two other aliphatic counterparts, compounds **2** and **3** (Fig. 2).

The total cellular uptake of compounds **5** and **6** was lower compared to uptake of compound **4**. Thus, from these large and aromatic amino acid conjugates *meta*-substituted L-phenylalanine derivative **4** enabled more efficient LAT1-mediated cellular uptake than with *para*-substitution (**5**) or with more flexible aliphatic L-lysine amino acid moiety (**6**). Moreover, uptake of these large, rigid and aromatic derivatives was independent of

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extracellular sodium or L-alanine and kinetic plots from the uptake curves were not biphasic, implying LAT1-selective cellular uptake (Fig. 2 and insets).

Curiously, for gabapentin and compounds **1** and **4** uptake kinetic plots revealed atypical/nonclassic kinetic behavior instead of linear fitting (Fig 2. Insets). This indicates sigmoidal auto-activation uptake profiles.

4. Discussion

To clarify the structural requirements for efficient and selective binding to and through human LAT1, competitive inhibition assays and cellular uptake studies were carried out *in vitro*. All tested compounds were able to bind selectively to LAT1 over LAT2 and ASCT2; all compounds reduced cellular uptake of [^{14}C]-L-leucine, but were unable to inhibit cellular uptake of [^{14}C]-L-alanine or [^{3}H]-L-glutamine significantly. Overall, *meta*-conjugation of L-phenylalanine increased binding to human LAT1 compared to *para*-conjugation or aliphatic amino acid moiety. Thus, our results from competitive inhibition studies are in accordance with our previous findings and previously published 3D-QSAR model of the rat LAT1-binding site (Gynther, et al. 2016; Peura, et al. 2011; Ylikangas, et al. 2014).

The known LAT1-inhibitor (Huttunen, et al. 2016a) was able to decrease cellular uptake of compounds which reached only moderate inhibition of [^{14}C]-L-leucine uptake (gabapentin, BCH) or had low total LAT1-mediated cell uptake (melphalan, compounds **2** and **6**). In contrast, compounds that inhibit the cellular uptake of [^{14}C]-L-leucine almost completely (compounds **1**, **4**, and **5**) were able to bind to/utilize LAT1 despite the presence of the inhibitor. This is conceivable, since these compounds have been designed based on the 3D-QSAR model of LAT1 binding site, and it is acknowledged that these compounds actually have greater affinity for LAT1 compared to the LAT1 inhibitor, which has an IC_{50} value of ~18 μM (Huttunen, et al. 2016a). Moreover, it has been reported that when inhibiting the

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uptake of these compounds via LAT1, they start to use another lower affinity but higher capacity transport mechanism, which can explain the increase in uptake of compounds such as **1** and **3** (Huttunen, et al. 2016b).

All compounds exhibited concentration-dependent cellular uptake (Fig. 2). Total cellular accumulation of the small size known LAT1-substrate gabapentin and transportable inhibitor BCH as well as of compound **7** was high compared to larger amino acid derivatives. Thus, it appears that BCH rather is a good substrate than an inhibitor for LAT1. From the larger compounds, ketoprofen derivative of *meta*-substituted L-phenylalanine (**4**), valproic acid derivative of *meta*-substituted L-phenylalanine with a methylene group between the amide bond and aromatic ring of L-phenylalanine (**1**) and *para*-substituted valproic acid derivative (**3**) enabled efficient cellular uptake. Total cellular uptake of these compounds was higher than of clinically used drug and known LAT1-substrate melphalan.

Based on the uptake studies, for a small and non-aromatic substituent, a methylene group modification between the amide bond and aromatic ring of *meta*-substituted L-phenylalanine (**1**) increased the LAT1-selectivity and transport efficacy. Similar results have been reported for example in rat brain *in situ* perfusion experiments, where methylene modification increased selective brain uptake of vaplroic acid prodrug via LAT1 (Gynther, et al. 2016). This suggests that a compound with the methylene group (**1**) could be better capable of fitting into the lipophilic pocket of the LAT1-binding site than a compound without this modification (**2**). Compound **2** as well as *para*-substituted L-phenylalanine derivatives compound **3** and melphalan started to utilize transporter(s) other than LAT1 for cell uptake at higher concentrations. For compound **2**, we were unable to specify this lower affinity transport mechanism, but we observed that it was not dependent on extracellular sodium or L-alanine. The cellular uptake of compound **3** decreased when extracellular sodium was removed, indicating that **3** was substrate also for sodium-dependent transporter(s). Moreover,

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L-alanine decreased cellular uptake of melphalan and in addition to LAT1, melphalan was a substrate for L-alanine sensitive transporter mechanism. In contrast, larger and more rigid aromatic substituent (**4**, **5** and **6**) enabled selective LAT1-mediated transport regardless of their amino acid moiety. The efficacy of cellular uptake varied: *meta*-substituted L-phenylalanine derivative enabled more efficient LAT-mediated transport than *para*-substitution or incorporation of more flexible and aliphatic L-lysine as amino acid moiety.

It has been previously stated that at some point, when the lipophilicity of a substituent increases sufficiently, such as in the case of *meta*-substituted L-phenylalanine derivatives, a compound acts more like an inhibitor rather than a substrate of LAT1 (Augustyn, et al. 2016; Uchino, et al. 2002). In this study, the total cellular uptake of large and lipophilic, efficient LAT1-selective compounds (**1** and **4**) was in the picomole range (pmol/mg of protein/min), whereas cellular uptake of L-leucine, natural substrate of LAT1, was three orders of magnitude higher (nmol/mg of protein/min). However, these kinds of compounds were still acting more like substrates rather than inhibitors. Thus, in contrast to previously published analysis performed with LAT1-substrates discovered by serendipity (del Amo, et al. 2008), it seems feasible to design compounds that act as LAT1 substrates, successfully compete with endogenous amino acids and utilize effectively LAT1 to gain cellular accumulation.

Our results confirm also that efficient binding to LAT1 does not necessarily correspond with efficient LAT1-mediated cellular uptake or *vice versa* (Huttunen, et al. 2016b). Instead, the speed of LAT1-mediated transport seems to vary with molecular size: the smaller substrates displayed higher uptake rates than larger compounds, as similarly the case with the thyroid hormones (Friesema, et al. 2001). In this study small size compounds, such as BCH and gabapentin, were unable to inhibit efficiently cellular uptake of [^{14}C]-L-leucine at 50 µM concentrations; instead they were quickly transported to the MCF-7 cells. Thus, method

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selection is of utmost importance when developing LAT1-utilizing compounds and in addition to binding studies, also cellular accumulation should be determined in future studies.

From a methological point of view, also selectivity of compounds between various membrane transporters should be determined by using cellular accumulation data, as such approach provides more accurate knowledge on selectivity than inhibition experiments alone. We were not able to detect unselectivity of melphalan and compounds **3** and **7** in inhibition studies, as these compounds did not inhibit cellular uptake of [^{14}C]-L-alanine or [^{3}H]-L-glutamine. However, extracellular L-alanine had an influence on cell uptake of compound **7** and melphalan, and extracellular sodium on cell uptake of compound **3**. Thus, these compounds could be transported to the cells by certain L-alanine sensitive or sodium-dependent transporter(s) without affecting the cell uptake of transporter´s natural substrates at one concentartion. Curiously, compounds **5** and **6** were able to increase the cellular accumulation of [^{14}C]-L-alanine into the cells to a statistically significant extent (Fig. 1b). The increase of cellular uptake of [^{14}C]-L-alanine may indicate that these compounds can bind to extra- or intracellular binding sites of LAT2 or other L-alanine transporting carriers. For example, it is known that LAT2 has a broader intracellular efflux-substrate selectivity range than LAT1 (Meier, et al. 2002). Therefore, it is likely that compounds **5** and **6** are transported into the cells in a LAT1-mediated manner and are bound to the intracellular binding site of LAT2 and thus, *trans*-stimulate the uptake of extracellular [^{14}C]-L-alanine.

The role of an unsubstituted carboxylic acid group for LAT1 binding is questioned and still controversial. Based on several previous studies, the carboxylic acid group should be unsubstituted in order to confer affinity for LAT1 (Geier, et al. 2013; Uchino, et al. 2002; Ylikangas, et al. 2014). In our own previous study, an isoleucine-quinidine ester prodrug displayed no affinity for LAT1 (Rautio, et al. 2015). However, two recent articles reported that the presence of an acidic functional group is not essential for LAT1-binding and for

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LAT1-substrates in *cis*-inhibition and *trans*-stimulation assays in LAT1 transfected human embryonic kidney (HEK) cells (Zur, et al. 2016) and in human derived cervix adenocarcinoma (HeLa S3) cells (Nagamori, et al. 2016). In these earlier studies, for example methyl and ethyl esters of L-leucine were substrates for LAT1. In the present study, the methyl ester of fluoro-L-tryptophan (**7**) displayed weak affinity for human LAT1 (13% inhibition, Fig. 1a), but it was transported efficiently to the MCF-7 cells (Fig. 2). However, cell uptake of compound **7** was unaffected by the presence of the LAT1 inhibitor despite the low binding to LAT1. Moreover, there was a L-alanine sensitive transporter mechanism involved in its cellular uptake in addition to LAT1. This indicates that if the carboxyl group of LAT1-substrate is derivatized to form for example a methyl ester, it can still maintain some affinity for LAT1 and could possibly be transported via LAT1 through cell membrane to some extent.

Kinetic plotting of concentration-velocity curves provide important insights from the cellular uptake mechanisms. The Eadie-Hofstee analysis revealed atypical auto-activation profile for LAT1-mediated cellular uptake for gabapentin as well as for compounds **1** and **4**. Atypical kinetics might indicate positive cooperativity between the multiple substrate bindings at the active binding site (Shapiro and Ling 1997; Shapiro, et al. 1999). This could occur after the binding affinity to the primary active site has been enhanced by binding of substrate to an allosteric site (Ueng, et al. 1997). This phenomenon has earlier been observed in drug-metabolizing enzymes (Atkins 2005; Galetin, et al. 2003) and drug transporters (Shapiro and Ling 1997; Shapiro, et al. 1999; Wagner, et al. 2017; Zhou, et al. 2007), particularly in those exhibiting broad substrate specificities (e.g., cytochrome P450 3A4 (CYP3A), multidrug resistance protein 1 (MDR1) and proton-stimulated organic cation transporter (plasma membrane monoamine tranporter (PMAT)) in a substrate-dependent manner. In previous *in vitro* studies in Madin-Darby canine kidney (MDCK) cells stably expressing PMAT, utilized

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for investigating the cellular uptake of serotonin, a typical linear kinetic plot was seen (Engel, et al. 2004). In contrast, the cellular uptake of metformin displayed atypical auto-activation kinetics (Zhou, et al. 2007). However, we do not have any evidence that there would be multiple binding sites in LAT1 and this possibility has not, to the best of our knowledge, been described by others either. Atypical kinetics observed for some compounds in this study was not detected for compound **4** in an earlier cellular uptake study in ARPE-19 cells (Puris, et al. 2017). This difference could be imputed to dissimilarity of cancer cells and healthy tissues, but the mechanism behind the atypical cellular uptake kinetics of LAT1-substrates is currently unclear and further studies are needed for clarifying it.

5. Conclusion

This study confirms that the *meta*-conjugation of L-phenylalanine increases binding to human LAT1 compared to *para*-conjugation or aliphatic amino acid moiety. More importantly, selective and efficient LAT1-mediated cellular uptake can be achieved with a large, rigid and aromatic *meta*-substituted L-phenylalanine derivative. For smaller, flexible and aliphatic conjugate, the presence of a methylene group between the aromatic ring of *meta*-substituted L-phenylalanine and the substituent may help to increase the LAT1-selectivity and transport efficacy. Furthermore, one of the most important finding of this study is methodological conclusion that can be drawn based on the results: when aiming at developing LAT1-utilizing compounds, it is of utmost importance to complement binding experiments with studies on cellular accumulation. Such integrated approach offers crucial information not only for transport efficiency but also for the selectivity of transport mechanisms that the compounds are able to utilize. Overall, our structural findings as well as the methodology used in this study may be exploited in future to design LAT1-utilizing compounds, such as markers for cancer imaging and (pro)drug molecules to enable more effective and safer treatments for cancer.

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Authorship contributions

Participated in research design: Kärkkäinen, Gynther, Laine, Rautio, Huttunen

Conducted experiments: Kärkkäinen

Contributed new reagents or analytical tools: Kärkkäinen, Gynther, Kokkola, Petsalo, Auriola, Lahtela-Kakkonen, Rautio, Huttunen

Performed data analysis: Kärkkäinen, Gynther, Huttunen

Wrote or contributed to the writing of the manuscript: Kärkkäinen, Gynther, Rautio, Huttunen

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Figure legends

Figure 1. The binding of compounds to LAT1, LAT2 and ASCT2 in MCF-7 cells. (a) The ability of compounds to bind to LAT1. The cells were co-incubated with compound at 50 µM or with a positive control at 1 mM and 0.157 µM [^{14}C]-L-leucine. (b) The ability of compounds to bind to LAT2. The cells were co-incubated with compound at 50 µM or with a positive control at 1 mM and 10 µM [^{14}C]-L-alanine. (c) The ability of compounds to bind to ASCT2. The cells were co-incubated with compound at 100 µM or with a positive control at 2 mM and 0.05 µM [^{3}H]-L-glutamine. Each point represents mean ± SEM, n = 3 and an asterisk denotes statistically significant difference from the control (***P<0.001, *P<0.05, one-way ANOVA, followed by Dunnett`s multiple comparison test).

Figure 2. Concentration-dependent cellular uptake of compounds with all active transport processes (solid black line with squares), when function of sodium-dependent transporters was inhibited by removing extracellular Na^+ (dashed line with circles) and when function of sodium-dependent transporters and LAT2 was inhibited by removing extracellular Na^+ and adding 2 mM L-alanine to uptake medium (dashed line with triangles). Insets, Eadie-Hofstee plots. Each point represents mean ± SEM and n = 3.

Figure 3. Cellular uptake of compounds at 10 µM concentration with or without the LAT1 inhibitor (100 µM) in MCF-7 cells. The data is presented as mean ± SEM, n = 6 and an asterisks denote a statistically significant difference from the respective control (*P < 0.05, **P < 0.005, ***P < 0.001, Student's t-test).

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Figure 1

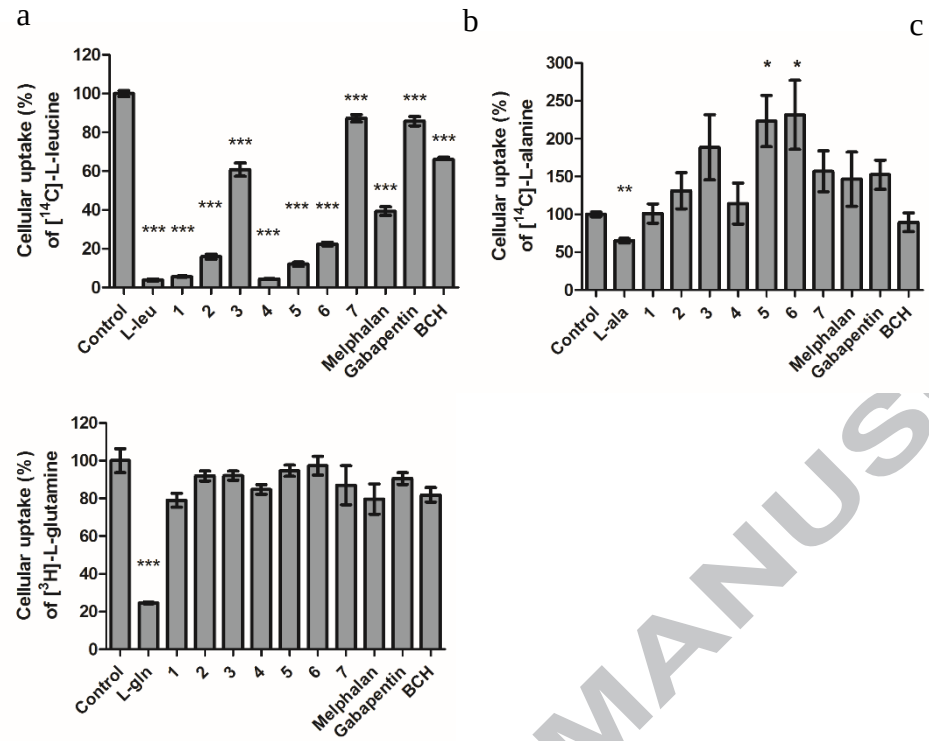

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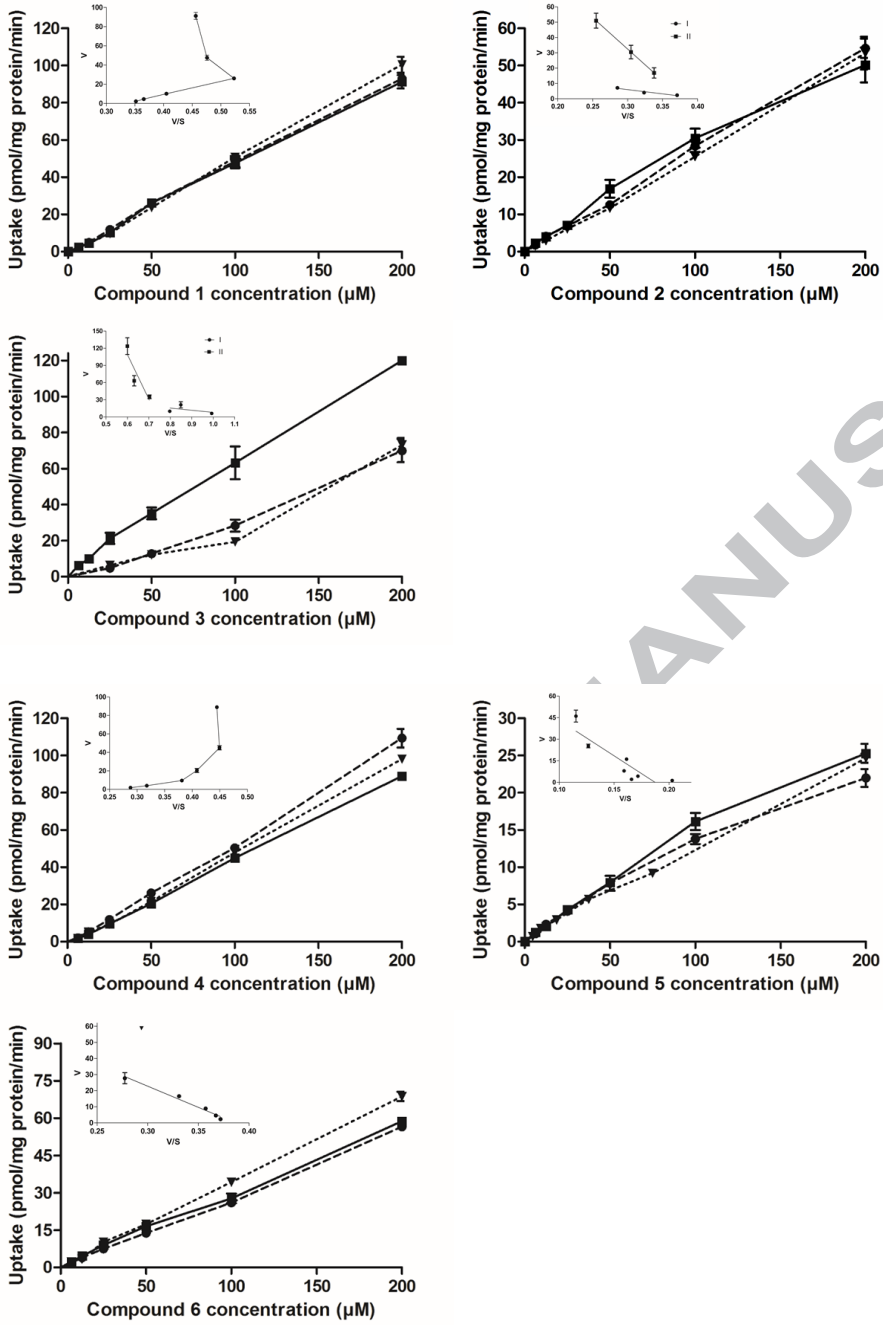
Figure 2



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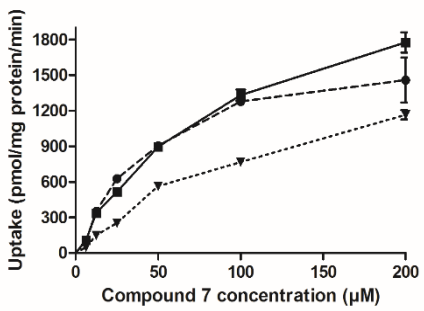

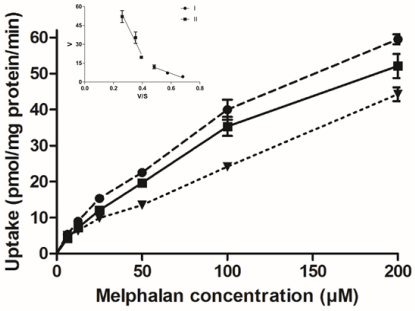

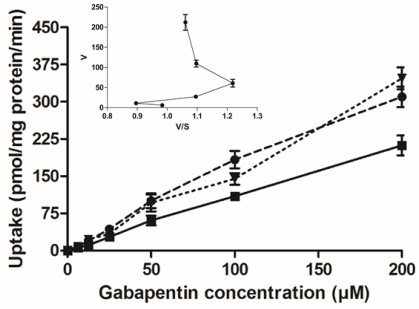

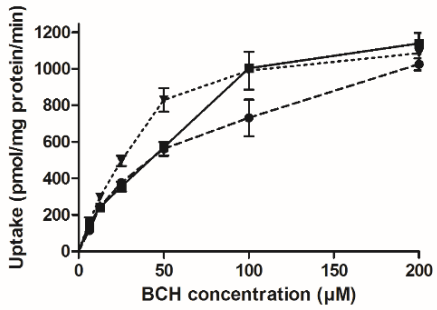

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Figure 3

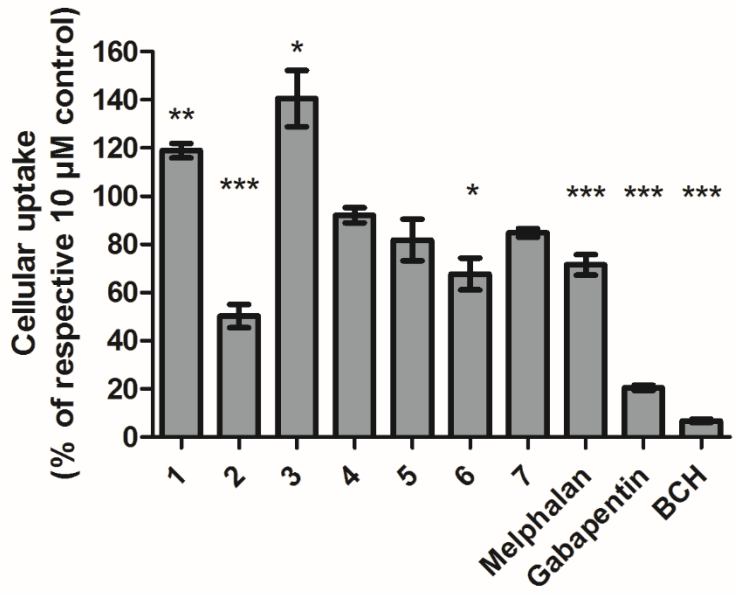

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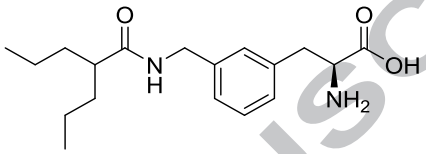
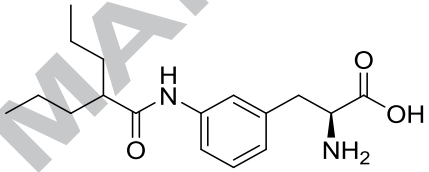
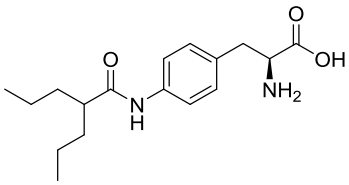
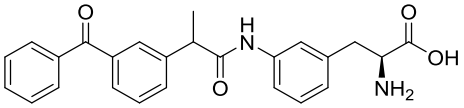
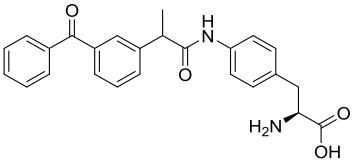
Table 1. Molecular structures (molecular weight) and ability of tested compounds to bind to LAT1.

Compound (MW)	Inhibition of cellular uptake of [^{14}C]-L-leucine, %[a]	Structure
1 (320)	94	
2 (306)	84±2	
3 (306)	40±6	
4 (416)	96	
5 (416)	88±2	

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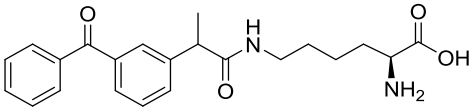
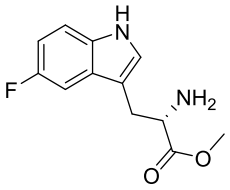
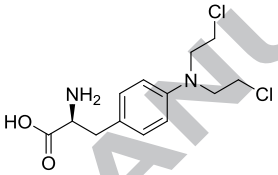
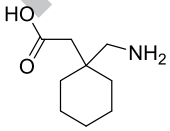
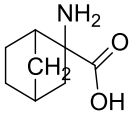
6 (383)	78±2	
7 (236)	13±3	
Melphalan (305)	61±4	
Gabapentin (171)	14±3	
BCH (155)	40±2	

[a] Inhibition % represent the average ± SD (n = 3) at a compound concentration of 50 μM.