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Amino acid ester prodrugs conjugated to the α-carboxylic acid group do not display affinity for the L-type amino acid transporter 1 (LAT1)

Jarkko Rautio *, Jussi Kärkkäinen, Kristiina M. Huttunen [1], Mikko Gynther [1]

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

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ABSTRACT

L-type amino acid transporter (LAT1) is an intriguing target for carrier-mediated transport of drugs as it is highly expressed in the blood–brain barrier and also in various types of cancer. Several studies have proposed that in order for compounds to act as LAT1 substrates they should possess both negatively charged α-carboxyl and positively charged α-amino groups. However, in some reports, such as in two recent publications describing an isoleucine–quinidine ester prodrug (**1**), compounds having no free α-carboxyl group have been reported to exhibit high affinity for LAT1 *in vitro*. In the present study, **1** was synthesized and its affinity for LAT1 was evaluated both with an *in situ* rat brain perfusion technique and in the human breast cancer cell line MCF-7 *in vitro*. **1** showed no affinity for LAT1 in either model nor did it show any affinity for LAT2 in an *in vitro* study. Our results confirm the earlier reported requirements for LAT1 substrates. Thus drugs or prodrugs with substituted α-carboxyl group cannot bind to LAT with high affinity.

1. Introduction

L-type amino acid transporter 1 (LAT1) is a sodium-independent heterodimeric transmembrane protein found in brain, testis and placenta. It is also known that the levels of functional LAT1 are also significantly up-regulated in the surface of several human tumor cells highlighting its essential role in the growth and proliferation of cells. LAT1 is responsible for transporting large neutral amino acids such as L-leucine, L-tryptophan, L-isoleucine and L-phenylalanine into cells (Kanai et al., 1998) but it can also carry amino acid-derived drugs such as levodopa, gabapentin, melphalan and baclofen (Cornford et al., 1992; Kageyama et al., 2000; van Bree et al., 1988; Wang and Welty, 1996). Furthermore, LAT1 has been demonstrated to be able to transport amino acid prodrugs, where the amino acids have been conjugated with drug molecules which are not LAT1 substrates as such (Gynther et al., 2008; Killian et al., 2007; Walker et al., 1994).

In previous studies it has seemed that in order to achieve efficient binding to LAT1, then the substrates should have an unsubstituted carboxylic acid or alternatively a negatively charged group as well as an unsubstituted amine functionality (Geier et al., 2013; Gynther et al., 2008; Uchino et al., 2002; Ylikangas et al., 2013). In these studies removal or the presence of substituents on either carboxylic acid or amine functionalities exerted a dramatic effect on the binding ability of these compounds resulting in only weak affinity or a complete inability to bind to LAT1. In addition, amino acid derivatives in which the carboxyl and primary amine groups were further away than 3 Å possessed only a modest affinity for LAT1 which was comparable to those compounds lacking either the negatively charged group or the amine group (Ylikangas et al., 2013).

However, some studies have indicated possible involvement of amino acid transport systems, including LAT1, in the transport of compounds lacking the free carboxyl group across cell membranes (Jain et al., 2004; Patel et al., 2013, 2014). For example, in two recent articles, an isoleucine–quinidine ester prodrug (**1** in Fig. 1) has been reported to exhibit high affinity for LAT1 both in human derived prostate cancer cells (PC-3)(Patel et al., 2013) and in the Madin–Darby canine kidney (MDCK–MDR1) cell line (Patel et al., 2014). In addition, affinity for LAT2 was reported (Patel et al., 2014). In this prodrug the isoleucine promoiety had been conjugated with quinidine from the carboxylic acid functionality which, as far as we are aware and according to the prevailing consensus, this modification should significantly reduce the affinity for LAT1. Since these results are at odds with the current knowledge, in the present study we synthesized the prodrug described by Patel et al. (2013, 2014) and investigated its affinity for LAT1 and LAT2. The LAT1 affinity experiments were carried out with two

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* Corresponding author. Tel.: +358 40 353 2791.
E-mail address: jarkko.rautio@uef.fi (J. Rautio).

[1] Authors contributed equally to this work.

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independent methods; *in situ* rat brain perfusion technique and in human breast cancer cell line MCF-7 *in vitro*, both of which have demonstrated their versatility in LAT1 affinity studies, (Gynther et al., 2008; Killian et al., 2007; Shennan et al., 2004) while LAT2 affinity was determined in MCF-7 cells.

2. Materials and methods

2.1. General synthetic methods

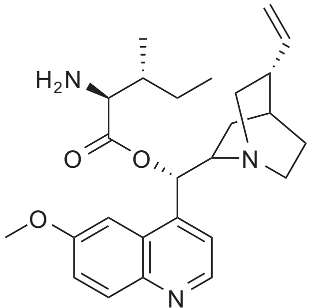
All the reactions were performed with reagents of commercial high purity quality without further purification. Reactions were monitored by thin-layer chromatography using aluminum sheets coated with silica gel 60 F_{245} (0.24 mm) with suitable visualization. Purifications by flash chromatography were performed on silica gel 60 (0.063–0.200 mm mesh). ^1H 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. pH-dependent NH-protons of the compounds were not observed. The final products were also characterized by mass spectroscopy with a Finnigan LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray ionization source and by elemental analysis (C, H, N) with a Perkin Elmer 2400 Series II CHNS/O organic elemental analyzer (Perkin Elmer Inc., Waltham, MA, USA).

2.2. Synthesis of isoleucine–quinidine (1)

The compound **1** was synthesized as previously described (Patel et al., 2013). ^1H NMR ($(CD_3)_2SO$): δ ppm 8.76 (d, $^3J_{HH} = 4.5$ Hz, 1H), 8.02 (d, $^3J_{HH} = 9.0$ Hz, 1H), 7.64 (d, $^3J_{HH} = 4.2$ Hz, 1H), 7.56–7.49 (m, 2H), 6.23–6.12 (m, 1H), 5.27–5.18 (m, 2H), 4.48–4.41 (m, 1H), 3.40 (s, 3H), 3.97–3.90 (m, 1H), 3.64–3.545(m, 1H), 3.51–3.41 (m, 1H), 3.31–3.20 (m, 2H), 2.78–2.69 (m, 1H), 2.45–2.38 (m, 1H), 2.19–2.10 (m, 1H), 2.05–2.00 (m, 1H), 1.87–1.79 (m, 2H), 1.59–1.50 (m, 1H), 1.46–1.37 (m, 1H), 1.32–1.21 (m, 2H), 1.04 (d, $^3J_{HH} = 5.7$ Hz, 3H), 0.88 (t, $^3J_{HH} = 7.3$ Hz, 3H) ; ^{13}C NMR ($(CD_3)_2SO$): δ ppm 168.13, 158.16, 147.26, 143.77, 139.72, 137.32, 131.39, 125.53, 122.31, 118.39, 117.25, 101.40, 71.68, 57.36, 56.02, 48.39, 47.79, 36.58, 35.31, 26.35, 23.49, 22.19, 19.27, 14.82, 11.29. MS (ESI$^+$) for $C_{26}H_{36}N_3O_3$ $(M + H)^+$: Calcd 438.58, Found 438.19. Anal. Calcd for ($C_{26}H_{35}N_3O_3 * 1.3$ TFA): C, 58.64; H, 6.25; N, 7.17; Found: C, 58.60; H, 5.85; N, 7.09.

2.3. In vitro uptake experiments in MCF-7 cells

MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine (2 mM), heat-inactivated fetal bovine serum (10%), penicillin (50 IU/ml) and streptomycin (50 Ag/ml). MCF-7 cells were seeded at the density of 1×10^5 cells/well onto collagen-coated 24-well plates. The cells were used for the uptake experiments one day after seeding. After removal of the culture medium, the cells were carefully washed with pre-warmed Na^+-free HBSS (Hank's balance salt solution) containing 125 mM chlorine chloride, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 5.6 mM glucose, and 25 mM HEPES (pH 7.4) and then pre-incubated in 500 µL of pre-warmed Na^+-free HBSS at 37 °C for 10 min before adding substrates for the uptake experiment. The cells were then incubated at 37 °C for 5 min in 250 µL of uptake medium: Na^+-free HBSS containing [^{14}C]-L-leucine or [^{14}C]-L-alanine. Subsequently, the cells were washed three times with ice-cold Na^+-free HBSS. 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). In our studies, K_m and V_{max} values were determined for [^{14}C]-L-leucine and [^{14}C]-L-alanine uptake under Na^+-free conditions. The K_m and V_{max} values were 141.0 ± 13.6 µM and 9.5 ± 0.28 nmol/mg protein/min for [^{14}C]-L-leucine, which are in agreement with those by Shennan et al. (2004) and >2 mM and 1.4 ± 0.6 nmol/mg protein/min for [^{14}C]-L-alanine, respectively. For the inhibition experiments the uptake of 0.157 µM [^{14}C]-L-leucine or 10.0 µM [^{14}C]-L-alanine was examined in the presence or absence of test compounds.



1

Fig. 1. Chemical structure of isoleucine–quinidine ester (**1**).

2.4. In situ rat brain perfusion technique

The ability of **1** to bind to rat LAT1 at the rat BBB was studied with the *in situ* rat brain perfusion technique, which has been described and validated earlier (Gynther et al., 2008). The 100% permeability-surface area (PA) product of [^{14}C]-L-leucine, a known substrate of LAT1, was determined with 30 s perfusion of 0.2 µCi/mL [^{14}C]-L-leucine (0.64 µM). The perfusion fluid was infused through the common carotid artery at the rate of 10 mL/min for 30 s using a Harvard PHD 22/2000 syringe pump (Harvard Apparatus Inc., Holliston, MA). In order to determine the interaction of **1** with LAT1 at the BBB, a 100 µM concentration of **1** was coperfused for 30 s with [^{14}C]-L-leucine. **1** was dissolved in the perfusion medium right before the perfusion, in order to avoid the release of isoleucine from the prodrug before the experiment. The PA product of [^{14}C]-L-leucine after coperfusion was compared with the 100% PA product of [^{14}C]-L-leucine. In the coperfusion studies the LAT1-binding of the investigated compound is shown by decreased PA product of [^{14}C]-L-leucine caused by a competitive binding to LAT1. All samples were analyzed for radioactivity by liquid scintillation counting (Wallac 1450 MicroBeta; Wallac Oy, Finland). Brain samples were dissolved in 0.5 mL of Solvable (PerkinElmer, Boston) overnight at 50 °C, and liquid scintillation cocktail (Ultima Gold, PerkinElmer, Boston) was added before the samples were analyzed. The perfusion medium consisted of a pH 7.4 bicarbonate-buffered physiological saline (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 $MgCl_2$ and 9 mM D-glucose). The solution was filtered, heated to 37 °C, and gassed with 95% O_2, 5% CO_2 to attain steady-state gas levels within the solution.

2.5. Animals

Male Wister rats 200–250 g, 7–8 weeks of age obtained from the National Laboratory Animal Centre (Kuopio, Finland) were used for *in situ* rat brain perfusions. Food and water was available ad libitum on a 12/12 h light/dark cycle with lights on at 6 am. Experiments were performed between 9:00 am and 16.00 pm in temperature and humidity-regulated rooms (22–24 °C, relative humidity: 60–70%). Rats were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and xylazine (5 mg/mL).