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ORIGINAL ARTICLE

System L amino acid transporter LAT1 accumulates O-(2-fluoroethyl)-L-tyrosine (FET)

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Abstract O-(2-fluoroethyl)-L-tyrosine (FET) labeled with fluorine-18 is an important and specific tracer for diagnostics of glioblastoma via positron emission tomography (PET). However, the mechanism of its quite specific accumulation in tumor tissue has not been understood so far. In this work we demonstrate that [^{3}H]L-tyrosine is primarily transported by the system L transporter LAT1 in human LN229 glioblastoma cells. FET reduced tyrosine transport, suggesting that it shares the same uptake pathway. More importantly, accumulation of FET was significantly reduced after siRNA-mediated downregulation of LAT1. *Xenopus laevis* oocytes expressing human LAT1 together with the glycoprotein 4F2hc (necessary to pull LAT-1 to the plasma membrane) exhibited a similar accumulation of FET as observed in glioblastoma cells. In contrast, no accumulation was observed in control oocytes, not overexpressing an exogenous transporter. Because LAT1 works exclusively as an exchanger of amino acids, substrates at one side of the membrane stimulate exchange against substrates at the other side. Extracellular FET stimulated the efflux of intracellular [^{3}H]L-leucine, demonstrating that FET is indeed an influx substrate for LAT1. However, FET injected into oocytes was not able to stimulate uptake of extracellular [^{3}H]L-leucine, indicating that FET is not a good efflux substrate. Our data, therefore, suggest that FET is trapped within cells due to the asymmetry of its intra- and extracellular recognition by LAT1. If also found for other transporters in tumor cells, asymmetric substrate recognition may be further exploited for tumor-specific accumulation of PET-tracers and/or other tumor-related drugs.

Keywords Transport · PET-tracer · Tyrosine · Tumor diagnostic · LN229 · Glioma

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Introduction

For the detection of malignant tumors, nuclear medicine takes advantage of radiolabeled compounds that accumulate in transformed cells to a much greater extent than in non-malignant cells. Due to their high metabolic rate, tumor cells avidly take up nutrients such as glucose and amino acids. Some ^{11}C- and ^{18}F-labeled amino acids have proven to be specific in brain tumor imaging. This specificity is ascribed to a high transport rate of the respective amino acid into tumor cells due to high expression of corresponding amino acid transporters, and in cases where labeled amino acids are incorporated in proteins, to a high rate of protein synthesis in the fast dividing tumor cells. Both, tracer transport and incorporation into proteins, are accepted as providing qualitative and quantitative information on tumor protein synthesis rate and thus proliferation in vivo.

Positron emission tomography (PET) reaches a high spatial and temporal resolution. With a half-life of about 110 min fluorine-18 is the preferred radionuclide used for clinical PET today (Coenen et al. 2010). An important ^{18}F-labeled amino acid is O-(2-[^{18}F]fluoroethyl)-L-tyrosine ([^{18}F]FET) (Langen et al. 2006; Pauleit et al. 2005). In animal experiments [^{18}F]FET exhibits low uptake in

non-neoplastic inflammatory cells and in inf l a m m a t o r y lymph nodes promising a higher specificity for the detection of tumor cells than other widely used compounds such as 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG) that exhibits high uptake and trapping also in normal brain tissues due to fast phosphorylation without further metabolism (Rau et al. 2002; Kaim et al. 2002).

Although several studies have shown that [^{18}F]FET is taken up into tumor cells by amino acid transporters, the transporter(s) responsible for its accumulation in these cells have not been identified so far. [^{18}F]FET is neither incorporated into proteins nor does it seem to be heavily metabolized (Langen et al. 2002; Wester et al. 1999; Pauleit et al. 2003). This leads to a higher in vivo stability of [^{18}F]FET compared to naturally occurring amino acids, simplifying image analysis. Accumulation of [^{18}F]FET in tumor cells depends thus mainly, if not exclusively, on transport (Langen et al. 2003). One would, therefore, expect that it requires an energy-consuming concentrative transport process. However, data today suggest that in most mammalian cells, [^{18}F]FET transport occurs via a Na^+-independent and thus energy-independent pathway (Heiss et al. 1999; Wester et al. 1999).

Of the numerous solute transporters that accept neutral amino acids as substrates, there are only a few that are Na^+-independent (Broer 2008; Broer and Palacin 2011; Hyde et al. 2003). Of these, the heteromeric system L amino acid transporter LAT1/4F2hc is highly expressed in fast dividing cells, including tumor cells. It accepts a wide range of large neutral amino acids including tyrosine (Broer 2008) and is, therefore, a good candidate to mediate [^{18}F]FET transport in tumors. In addition, LAT1/4F2hc works as an obligatory exchanger (antiport) by coupling the influx of an extracellular to the efflux of an intracellular substrate. That way, concentration of an intracellular exchange substrate by a Na^+-dependent transporter could provide the driving force for [^{18}F]FET accumulation. However, using *Xenopus laevis* oocytes as expression system, a recent study found that FET is only a poor uptake substrate for LAT1 (Lahoutte et al. 2004). Based on this study on one hand, and [^{18}F]FET accumulation studies with rat F98 glioma cells on the other hand (Pauleit et al. 2005), others have concluded that [^{18}F]FET may be selectively transported via LAT2/4F2hc (Langen et al. 2006). However, a subsequent study performing competition studies on *X. laevis* oocytes expressing mouse LAT2/4F2hc found a rather low affinity of LAT2 for FET (Makrides et al. 2007).

The aim of the present study was thus to elucidate the transporter(s) responsible for [^{18}F]FET transport into LN229 human glioblastoma cells and to shed light on the mechanisms of its accumulation. The knowledge of these processes will aid future approaches to optimize the specificity of further PET tracers for tumor diagnostic.

Materials and methods

Cell culture

The human glioblastoma cell line LN229 was obtained from ATCC (CRL-2611). They were grown in Dulbecco's modified Eagle's medium, supplemented with 4 mM glutamine and 10 % fetal bovine serum (at 37 °C, 10 % CO_2). Cells were regularly tested for mycoplasma infection using 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, Roche Molecular Biochemicals, Mannheim, Germany). No contamination was detected.

Transport studies in LN229 cells

Transport studies were carried out in cells grown to confluence in 96-well plates. All amino acids used were L-isomeres. Cells were washed twice with Locke's solution (LS: 154 mMNaCl5.6 mMKCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 3.6 mM $NaHCO_3$, 5.6 mM glucose, pH 7.4) and preincubated for 15 min in LS containing 50 µM tyrosine (100 µl/well). Cells were then incubated for 30 s at 37 °C in the same solution containing in addition 5 µCi/mlL-[ring-3,5-^{3}H]tyrosine (42.6 Ci/mmol, Perkin Elmer), further named [^{3}H]tyrosine and a competing was indicated amino acid (1 mM). The cells were then immediately washed three times with ice-cold LS and lysed in 0.5 M NaOH (50 µl/well, 45 min at room temperature). After neutralization of the lysates with 50 µl of 0.5 M HCl and 50 µl LS, 100 µl of each sample was measured by liquid scintillation counting (TRI-CARB 1900 CA, Packard). The background was determined by addition of LS containing 50 µM [^{3}H]tyrosine(5 µCi/ml) followed by immediate washing steps and subtracted from all values. Radioactivity measured in each sample was related to its protein content determined using BCA Protein Assay Kit (Pierce).

Quantitative reverse transcription and polymerase chain reaction (qRT/PCR)

Total RNA was isolated from confluent LN229 cells using the RNeasy Mini Kit (Qiagen) and quantified by the absorption at 260 nm. One-Step qRT/PCR was performed with the Quanti Tect RT/PCR Kit (Qiagen) in 25 µl reactions in a 96-well spectrofluorometric thermal cycler (iCycler, Bio-Rad) (dNTPs: 400 µM each, $MgCl_2$: 5.3 mM, cycle: 94 °C 15 s, 60 °C, 60 s). Fluorescence was monitored at each 60 °C annealing/extension step. Oligonucleotides and Taqman probes were described before (Rotmann et al. 2007), except for LAT1 and 2: hLAT1.660ss (GAAGGGTG ATGTGTCCAATCTA), hLAT1.796as (TTCTGTAGGGG TTGATCATTTC), LAT1Taq1 (CAACTTCTCATTTGAAG GCACCAAACTG); hlAT2.1431ss (TGACTTCATTGAGC

TGCTAACCC), hLAT2.1530as (GTCCTCATTAGCCTCC TCTGTCC), hlAT2.Taq1 (TGAGCCAGAAGATGTGTG TGGTCGTGTAC).

LAT1 knock down in LN229 cells

200,000 LN229 cells per 6 well were seeded 12 h before transfection. For each well 15 n Mol si RNA against hLAT1 (ss: CUCUUUGCCUAUGGAGGAU, Sigma-Aldrich) or non-target RNA (Eurogentec SR-CL000-005) were incubated with 112.5 µl OPTI-MEM (Life Technologies). At the same time 10 µl Saint Red (Synvolux Therapeutics) was incubated with 102.5 µl OPTI-MEM. After 5 min, the two preparations were combined and after further 10 min supplemented with 675 µl cell culture medium without serum. LN229 cells were incubated with this mixture for 3 h and then 2 ml medium supplemented with 10 % FBS was added. 24 h later a second transfection was performed as described for the first. After further 24 h, cells were washed twice with LS and then either lysed for Western blot analyses or processed for HPLC analyses (see below).

Western blot analysis

Cells were lysed with 0.15 ml radio immune precipitation assay buffer (RIPA: 1 % deoxycholate, 1 %Triton-x-100, 0.1 % SDS, 150 mM NaCl, 2 mM $MgCl_2$, 10 mM Tris-HCl pH 7.2) containing protease inhibitors (Complete Mini EDTA-free protease inhibitor tablets, Roche, Basel) for 1 h at 4 °C and centrifuged for 20 min at 1,400 rpm. An aliquot of each supernatant (protein content: 10 µg) was mixed directly with an equal volume of 2 × sample buffer (125 mM Tris base, 20 % glycerol (v/v), 5 % SDS, 0.001 % bromophenol blue, 8 M urea, 2 % mercaptoethanol (v/v)) and incubated for 10 min at 37 °C.

Lysates were separated by 10 % SDSPAGE and then blotted onto nitrocellulose membranes (Protran 83, Whatman, Maidstone). Staining for hLAT1 proteins was achieved by sequential incubations in Blotto (50 mM Tris pH 8, 2 mM $CaCl_2$, 0.01 % antifoam A [Sigma–Aldrich], 0.05 % Tween 20 and 5 % nonfat dry milk) supplemented with 5 % goat serum for 2 h at room temperature; three times for 10 min each in antibody dilution buffer (5 % BSA, 0.1 % Tween 20 in TBS [10 mM Tris-HCl, pH 8 and 150 mM NaCl] pH 7.4), a 1:5000 dilution of rabbit anti-hLAT1(Cell Signaling 5347) overnight at 4 °C; three times in Blotto for 10 min at room temperature; a 1:10000 dilution of peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Calbiochem 401393) in Blotto for 1 h at room temperature; three times in TBS-T (10 mM TrispH 8, 150 mM NaCl, 0.05 % Tween 20); once in TBS; and finally in chemiluminescence reagent (Western Lightning ECL-Plus, Perkin Elmer) for 1 min. Chemiluminescence films were then immediately exposed to the membranes. For standardization, membranes were stained with anti GAPDH (14C10) (Cell Signaling monoclonal antibody 2118) (1:1000) and the same secondary antibody as before. Protein bands were quantified using Chemi Doc and the Quantity One software (BioRAD).

Transport studies in *X. laevis* oocytes

Expression of cRNA in X. laevis oocytes

The coding region of LAT1 was amplified by PCR using pcDNA 3.1(+)_hLAT1 (a generous gift from Stefan Broer, ANU Canberra, Australia) and the sense and antisense oligonucleotides LAT1 SacII (TCCCCGCGGATGGCGGGTG CGGGCCCGAAGCGGCGCG) and LAT1 ClaI (GGCATC GATCTATGTCTCCTGGGGGACCACCTGCATG), respectively, as primer. The coding region of LAT2 was amplified by RT/PCR using total RNA from human peripheral blood mononuclear cells as template and the sense and antisense oligonucleotides hLAT2.723fwd (GAGGTTG-CAATCCTACTGAGAAGG) and hLAT2.2492rev (ACC-CACACCAAAGTCCTACCAC) as primers. Both cDNAs were inserted in the pSGEM Vector (Hoffmann et al. 2006) giving rise to the plasmids hLAT1- and hLAT2-pSGEM, respectively. 4F2hc in pGHJsC2 (H4F2FL pGHJsC2) was also a gift from Stefan Broer. Plasmids were linearized as follows: hLAT1 with PacI, hLAT2 with NheI and 4F2hc with AccI. cRNAs were prepared by in vitro transcription from the T7 promoter (mMessagem Machine in vitro transcription kit, Ambion, AMS Biotechnology). 10 ng of 4F2hc and 20 ng of LAT1or -2 cRNA in 20 nl water were injected in each *X. laevis* oocyte (Dumont stages V and VI). Non-injected oocytes were used as controls. All experiments were performed 2 days after cRNA injection.

Leucine uptake

Oocytes were washed three times in ice-cold uptake solution (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, and 5 mM Tris, pH 7.5) and then transferred to the same solution supplemented with 50 µM leucine and 10 µCi/ml L-[4,5- ^{3}H]leucine (54.1 Ci/mmol, Perkin Elmer, further named [^{3}H]leucine)leucine and where indicated 1 mM FET. After 15 min incubation at 20 °C, oocytes were washed 5 times with ice-cold uptake solution and solubilized individually in 2 % SDS. The radioactivity of the lysates was determined in a liquid scintillation counter. To assess intracellular trans-stimulation, oocytes were kept in uptake solution without any amino acids for 2 days, with buffer changes every 12 h. Oocytes were then injected with either 40 nl water, 10 mM FET in water or 10 mM leucine. Immediately after, uptake of [^{3}H]leucine was measured as described above.

Leucine efflux

Oocytes were washed three times in ice-cold uptake solution and incubated in uptake solution for 6 h at 18 °C. Each oocyte was injected a second time with 40 nl of 10 mM [^{3}H]leucine (100 µCi/ml). The oocytes were then immediately transferred into uptake solution containing either 1 mM FET, 1 mM Leu or no amino acids. After 30 min incubation at 20 °C, the radioactivity in the extracellular buffer was determined by liquid scintillation counting.

Quantification of amino acids by high-performance liquid chromatography

FET, tyrosine and leucine were measured by high-performance liquid chromatography (HPLC) using precolumn derivatization and fluorescence detection.

LN229 cells were preincubated in LS at 37 °C, 3 × 10 min with buffer changes in between and then either washed immediately or incubated for 2 or 4 h in LS alone or in LS containing either leucine, FET or tyrosine (0.1 mM). Subsequently, cells were washed twice with ice-cold PBS (0.14 M NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4), and lysed in 500 µl ice-cold 70 % Et OH, supplemented with 2 nmol homoserine as external standard.

Xenopus oocytes (two each) were washed twice in ice-cold uptake solution and then transferred in 200 µl uptake solution containing no amino acids or 0.1 mM of either leucine, FET or tyrosine. Oocytes were then either washed immediately or incubated for 4 h in the respective amino acid solution. Washing steps were repeated 5 times with ice-cold uptake solution. Oocytes were then lysed in 0.2 ml ice-cold 70 % Et OH supplemented with 2 nmol homoserine (30 min, 4 °C).

Samples were centrifuged at 14,000 × *g* for 10 min. Supernatants were dried by vacuum centrifugation, resuspended in 150 µl 0.5 M borate buffer pH 10 and purified using 10 K Omega centrifugal devices (Pall Nano sep). 50 µl of these sample were incubated with 12 µl OPA reagent (30 mg o-phthaldialdehyde, 2.7 ml methanol, 300 µl 0.5 M borate buffer pH 10, 30 µl 2-mercaptoethanol) and 20 µl acidic acid (1 M) for 180 s. Amino acid derivatives (15 µl sample each) were separated on a Nova-Pak column (C18, 4 µm 3.9 × 300 mm, Waters, Eschborn, Germany) using a gradient of 50 mM sodium acetate, pH 6.8 supplemented with 0.044 % triethylamine (0′: 87 %; 10′: 85 %; 16′: 84 %; 39′: 71 %, 40′: 64 %; 42′: 0 %; 47′: 0 %; 49′: 87 %) and acetonitrile (complementary to 100 %, respectively, flow rate 1 ml/min). Fluorescence (excitation wavelength, 330 nm; emission wavelength, 450 nm) was monitored with a Bischoff 8,470 fluorimeter and quantified using the analysis program McDAcq (Bischoff, Leonberg, Germany). The cell volume was determined using a Scepter Cell Counter, Millipore.

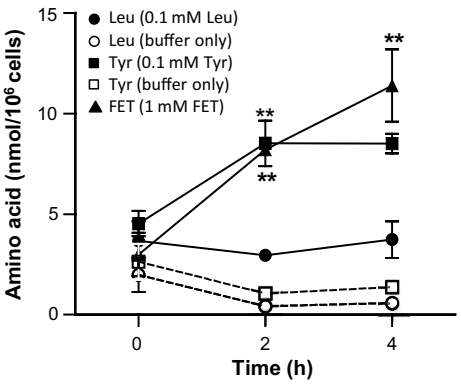

Fig. 1 FET and tyrosine accumulate in human LN229 glioblastoma cells. Confluent cells were pre-incubated three times for 10 min in Locke's solution (LS) and then for 0, 2 or 4 h in LS containing either 0.1 mM Leu (*black circles*), Tyr (*black squares*) or FET (*black triangles*). Cells were then washed, lysed and their content of the respective amino acid they had been incubated in was determined by HPLC. Control cells were incubated in LS alone (*dotted lines*) and their content of Leu (*open circles*) and Tyr (*open squares*) was determined by HPLC. *Data points* represent mean ± SEM ($n = 2$–3). Statistical analysis was performed using one-way Anova with Bonferroni post test. *Two stars* represent $p \leq 0.01$, no labeling corresponds to $p > 0.05$, of FET or Tyr compared to Leu content at the given time point

Results

The system L amino acid transporter LAT1 is responsible for FET accumulation into human glioblastoma cells

Our study was designed to elucidate which transporter is involved in [^{18}F]FET uptake and accumulation in human LN229 glioblastoma cells. HPLC analyses revealed a strong increase in intracellular FET concentrations over 4 h in these cells exposed to buffer containing 100 µM FET (Fig. 1). Taking cell number and volume (on average 2.8 ± 0.1 pl) into account, the intracellular FET concentration after 4 h reached 3.9 ± 0.65 mM. Because FET is a tyrosine derivative, we also studied tyrosine accumulation and found that it occurred to a similar extent as that of FET (Fig. 1). In contrast, intracellular concentrations of leucine, a neutral amino acid that shares several amino acid transporters with tyrosine, hardly changed upon incubation of LN229 cells in buffer containing 0.1 mM leucine. We next wondered if FET and tyrosine share the same transport pathways. In fact, uptake of 50 µM [^{3}H]tyrosine was reduced to 37.7 ± 0.3 % in the presence of 1 mM FET (Fig. 2a). Tyrosine transport was completely independent of the presence of Na^+ ions (Fig. 2b) and inhibited by leucine,

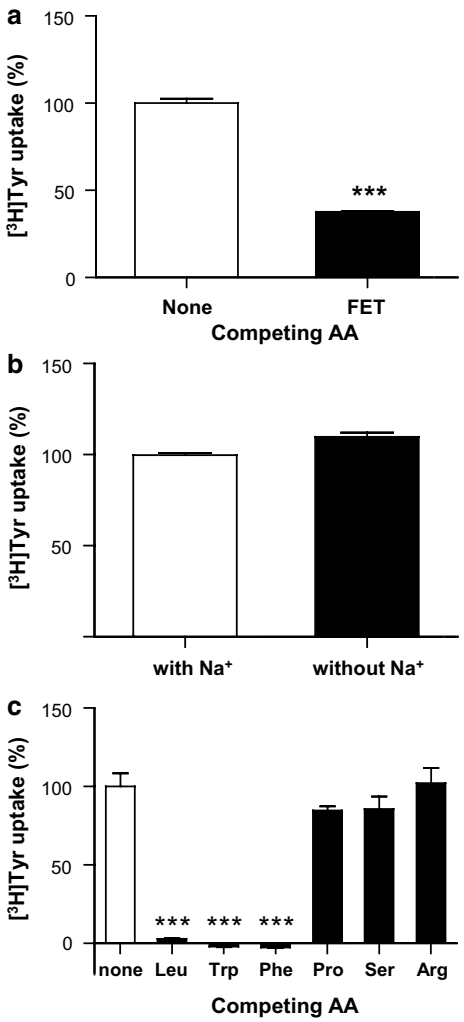

Fig. 2 FET competes with tyrosine for uptake into LN229 glioblastoma cells via system L. Confluent cells were washed and then exposed to buffer containing 50 µM [^{3}H]tyrosine (5 µCi/ml) for 30 s in the absence or presence of 1 mM of the indicated competing amino acid (**a**, **c**) or in the absence or presence of Na^+ (**b**). Cells were then washed in ice-cold buffer and lysed. The radioactivity of the lysates was determined by scintillation counting. The background radioactivity derived from arginine bound to the cells (determined by addition of LS containing 50 µM [^{3}H]tyrosine (5 µCi/ml) followed by immediate washing steps) was subtracted from all values. For each cell batch, the mean of the values from cells under control conditions (Na^+-containing buffer, no competing amino acid) were set 100 %. Columns represent mean ± S.E.M (n = 8–32). Statistical analyses were performed using t test (**a**, **b**) or one-way ANOVA with Bonferroni's posttest (**c**). *Three stars* represent $p \leq 0.001$; no labeling corresponds to $p > 0.05$, compared to control conditions, respectively

tryptophane and phenylalanine, but not by proline, serine or arginine (Fig. 2c). This suggests that tyrosine is taken up by the system L transporter LAT1 in LN229 cells and that FET shares the same transport system. Of the other transporters known to accept tyrosine as a substrate, $ATB^{0,+}$ and B^0AT1 are Na^+-dependent, LAT2 is inhibited by serine and TAT1

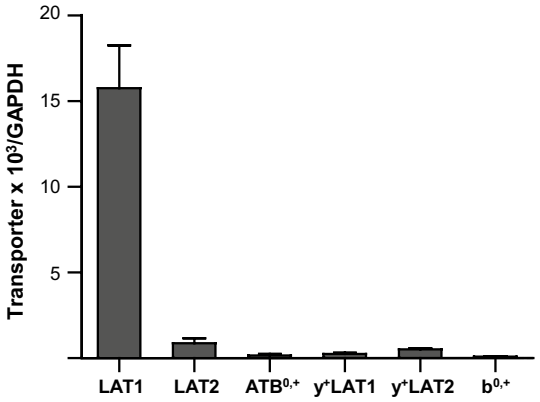

Fig. 3 System L amino acid transporter LAT1 is the predominantly expressed in LN229 glioblastoma cells. Total RNA was extracted from confluent LN229 cells, and expression of mRNAs encoding the indicated neutral amino acid transporters known to accept tyrosine as a substrate, was quantified via qRT-PCR. GAPDH (glycerine-aldehyde-3-phosphate-dehydrogenase) was used as control for relative measurements. *Columns* represent mean ± S.E.M ($n = 6$)

is not inhibited by leucine. Furthermore, $b^{0,+}AT$, y^+LAT1 and y^+LAT2 can be excluded because they are inhibited by arginine, LAT3 and LAT4 because they are not inhibited by tryptophane.

Profiling the mRNA expression of transporters accepting a broad range of neutral amino acids as substrate revealed a very strong expression of LAT1 in these cells (Fig. 3). Assuming a similar efficiency of the qRT/PCR (Rotmann et al. 2007), the mRNA expression of all other transporters was at least tenfold lower. This supports the notion that tyrosine and FET are predominantly transported by LAT1 in LN229 cells. However, is LAT1 also the transporter responsible for FET accumulation? To address this question, we down-regulated LAT1 in LN229 cells using siRNA. LAT1 protein diminished substantially in cells transfected with siRNA directed specifically against LAT1, compared to untransfected cells or cells transfected with non-target RNA (Fig. 4a, b). After incubation in 100 µM FET, cells with down-regulated LAT1 had accumulated significantly less FET compared to the respective control cells (Fig. 4c).

LAT1 mediates FET accumulation in *X. leavis* oocytes

Although our data in LN229 cells strongly suggest that FET accumulates in these cells due to transport mediated by LAT1, we could not formally proof that FET is a LAT1 substrate and that LAT1 is sufficient for FET accumulation. To address this question, we expressed LAT1 in *X. laevis* oocytes together with the glycoprotein 4F2hc, necessary for targeting LAT1 to the plasma membrane.

As observed in LN229 cells, FET and tyrosine, but not leucine, accumulated in LAT1-expressing oocytes exposed to buffer containing 100 µM of the respective amino acid

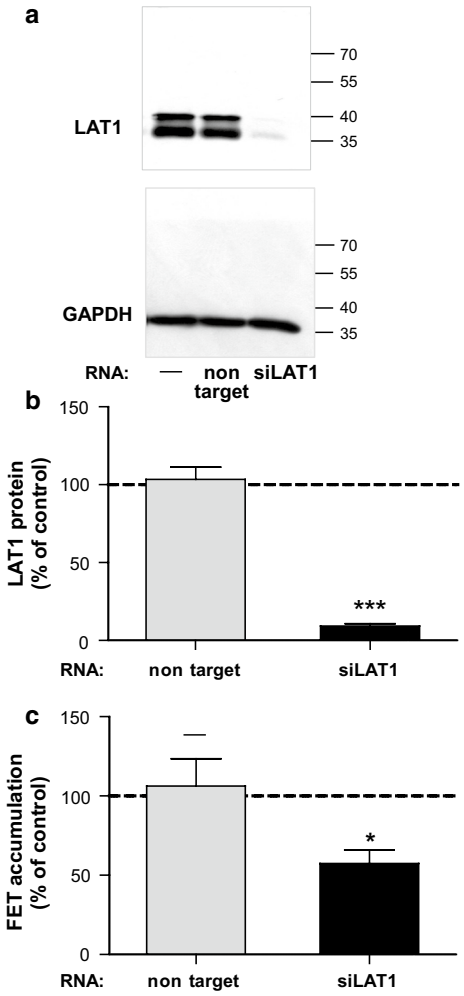

Fig. 4 Down-regulation of LAT1 leads to diminished FET accumulation in LN229 glioblastoma cells. LAT1 was down-regulated by transfecting LN229 cells at two consecutive days with an anti LAT1 siRNA. Cells were examined 24 h after the 2nd transfection. Untransfected cells and cells transfected with a non-target RNA were used as controls. **a** Representative Western blot detecting LAT1 and GAPDH proteins. The latter was examined as control for equal loading of individual gel lanes. **b** Quantification of LAT1 protein relative to GAPDH. **c** Cells were pre-incubated three times for 10 min in LS and then for 2 h in LS containing 0.1 mM FET. Cells were then washed, lysed and their amino acid content was determined by HPLC. **b**, **c** Values (mean ± S.E.M.) are expressed as percent of non-transfected control cells (*dashed line*), (n = 3–4). Statistical analyses were performed using t test. *One* and *three stars* represents $p \leq 0.05$ and ≤ 0.001, respectively, compared to cells transfected with non-target RNA

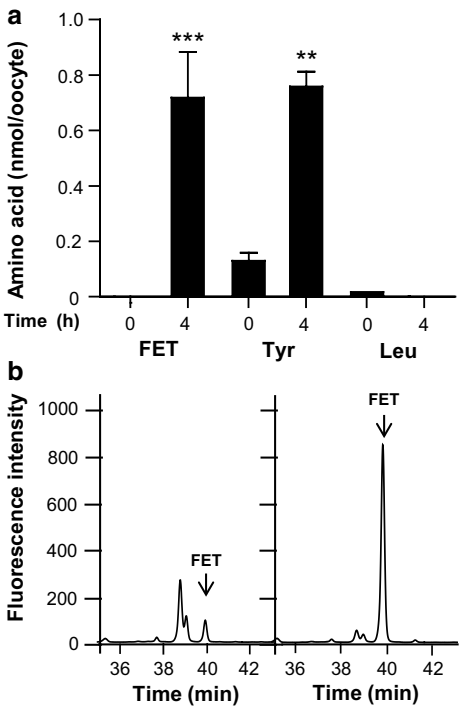

Fig. 5 FET accumulates in *Xenopus laevis* oocytes expressing LAT1. **a** *Xenopus laevis* oocytes were injected with cRNA encoding human LAT1 and the glycoprotein 4F2hc, necessary for localization of LAT1 in the plasma membrane. Transport experiments were performed two days later. Oocytes expressing human LAT1/4F2hc or no exogenous transporter were transferred to buffer containing either 0.1 mM Leu, 0.1 mM FET or 0.1 mM Tyr and either processed immediately (0 h) or incubated for 4 h, as indicated. The intracellular amounts of the respective amino acid were determined by HPLC. Values obtained from oocytes without exogenous transporter were subtracted from the corresponding value obtained from LAT1/4F2hc-expressing cells. *Columns* represent mean ± S.E.M (n = 3–4). Statistical analysis was performed using one-way ANOVA with Bonferroni's post test. No labeling, *two stars* and *three stars* correspond to $p > 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively, comparing the indicated amino acid at 0 and 4 h, respectively. **b**, **c** Representative HPLC diagram obtained with lysates from LAT1-expressing oocyte incubated for 0 h (**b**) and 4 h (**c**) in 0.1 mM FET, respectively

for 4 h (Fig. 5). Estimating an intracellular water volume of about 400 nl for stage 5 and 6 oocytes (Taylor and Smith 1987), the LAT1-mediated increase in intracellular FET and tyrosine concentrations after 4 h was 1.8 ± 0.4 and 1.9 ± 0.1 mM, respectively (calculated by subtracting values obtained from oocytes not expressing an exogenous transporter from those expressing LAT1). In contrast, intracellular leucine concentrations did not increase upon incubation in buffer containing 100 µM leucine. Also, in oocytes not overexpressing an exogenous amino acid transporter, intracellular concentrations measured after 4 h incubation in buffer containing 100 µM FET (218 ± 13 µM), tyrosine (366 ± 65 µM), or leucine (276 ± 48 µM) were not significantly different from those measured for the respective amino acid at 0 h. These experiments demonstrate that LAT1 is both necessary and sufficient for intracellular accumulation of FET.

FET is an influx, but not a good efflux substrate of LAT1

How can the difference in intracellular accumulation between leucine, a well-known substrate of LAT1, and FET

be explained? Further experiments with LAT1-expressing *Xenopus* oocytes showed that FET is in fact an uptake substrate for LAT1. As observed in LN229 cells, FET inhibited LAT1-mediated transport also in oocytes. The uptake of 50 μM [^{3}H]leucine was reduced to 40 ± 4.5 % in the presence of 1 mM FET (Fig. 6a). More importantly, 1 mM extracellular FET stimulated efflux of [^{3}H]leucine previously injected into the oocytes, to the same extent as 1 mM extracellular leucine (Fig. 6b). This demonstrates that FET is taken up into the oocytes in exchange with intracellular leucine. We next investigated if FET is also an efflux substrate for LAT1 by testing if it trans-stimulates leucine uptake into oocytes, depleted of intracellular amino acids beforehand. In contrast to leucine, FET injected into such oocytes (each to give a final intracellular concentration of about 1 mM) was not able to trans-stimulate [^{3}H]leucine influx, indicating that FET is not a good efflux substrate for LAT1 (Fig. 6c).

Discussion

In the present study, we addressed the question which transporter is responsible for accumulation of FET in human LN229 glioblastoma cells. Experiments in these cells as well as in *X. laevis* oocytes expressing the human neutral amino acid transporter LAT1 (endogenously and exogenously, respectively) demonstrate that this transporter is both necessary and sufficient for FET accumulation.

These data are apparently in contradiction with a previous study showing that FET is only a poor uptake substrate for LAT1 (Lahoutte et al. 2004). In this study, 0.1 mM extracellular FET trans-stimulated efflux of leucine from LAT1-expressing *X. laevis* oocytes to a much lesser extent than 0.1 mM extracellular phenylalanine. However, efflux into FET-containing buffer was about tenfold higher then efflux into buffer not containing any amino acid. Therefore, this study also demonstrates that FET is an uptake substrate for LAT1. The lower trans-stimulation in comparison with phenylalanine is probably due to a low affinity of LAT1 for FET. In line with this is our observation that at an extracellular concentration of 1 mM, FET and leucine trans-stimulated LAT1-mediated efflux equally well. Also, 1 mM FET inhibited uptake of either 100 μM tyrosine in LN229 cells or of 100 μM leucine in LAT1-expressing *X. laevis* oocytes only partially (by about 60 % in both cases), while leucine, tryptophane and phenylalanine inhibited LAT1 in LN229 cells virtually completely. Together these data suggest that FET is a low-affinity uptake substrate for LAT1.

In contrast, no trans-stimulation could be observed when FET was supplied intracellularly at a concentration of 1 mM, suggesting that FET is either not an efflux substrate at all or that it is recognized with extremely low affinity by LAT1. This indicates asymmetrical intra- and extracellular recognition of FET by LAT1. Such an asymmetry has also been described for other LAT1 substrates with leucine and methionine being better efflux then influx substrates for example (Meier et al. 2002; Verrey 2003). Interestingly, we also found a pronounced LAT1-mediated accumulation of tyrosine in both, LN229 cells and *X. laevis* oocytes, suggesting that the intracellular affinity of LAT1 for tyrosine is also low.

In a previous study with human cationic amino acid transporters (hCAT), that are also Na^+-independent exchangers, we found that the expression of the low-affinity isoform (hCAT-2A) in *X. laevis* oocytes leads to much higher intracellular substrate concentrations then the expression of the high-affinity hCAT-1 (Closs et al. 1997), although substrate recognition by these transporters is most likely symmetrical at the two sides of the membrane. What matters here is the low intracellular affinity. The latter must have an even more pronounced effect when substrate recognition at the outside is superior in comparison with the inside, as seems to be the case for LAT1 and FET. Our data suggest, therefore, FET to be trapped inside LAT1-expressing cells due to the lack or very poor efflux. Trapping or asymmetrical transport of FET has already been suggested by an earlier study showing linear unidirectional transport for both, tumor and normal brain tissue, however, to a much lesser extent in the latter (Wester et al. 1999).

Although [^{18}F]FET is not incorporated into proteins, intracellular metabolism to another membrane impermeable derivative could also lead to tracer accumulation. A previous pharmacokinetic study found a low amount of [^{18}F]FET metabolites in human plasma within the first 2 h after intravenous application (Pauleit et al. 2003). This indicates an overall slow metabolism of FET, but does not exclude trapping of intracellular metabolites. Our HPLC analyses of intracellular FET in LN229 cells and *X. laevis* oocytes demonstrate now that it is indeed FET itself, and not a metabolite of it, that accumulates within these cells. In addition, our trans-stimulation experiments, where we injected FET into oocytes and measured exchange against [^{3}H]leucine immediately after, support the notion that FET does not need to be metabolized to be trapped within cells.

At a radio-pharmacological dose of 10 mCi per patient, resulting in a plasma concentration of less than 3 nM [^{18}F]FET (neglecting clearance), only very few [^{18}F]FET molecules should be taken up per LAT1 transporter molecule, e.g. considering the presence of an excess of competing neutral amino acids in the cerebrospinal fluid. High transporter expression in comparison with the surrounding tissue may, therefore, be a prerequisite for the specific labeling of tumor cells with FET. After down-regulation of LAT1 in LN229 cells, FET accumulation was reduced, demonstrating that transporter copy number matters.

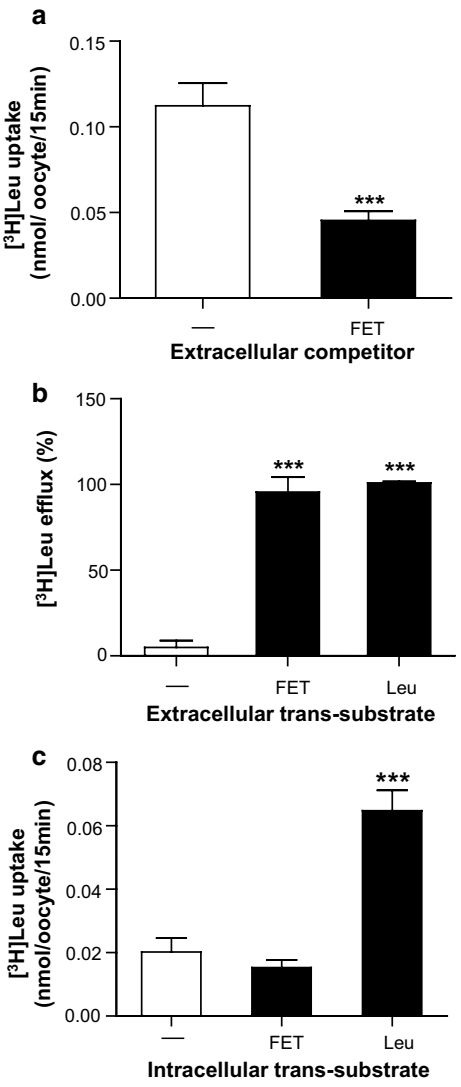

Fig. 6 1 m MFET trans-stimulates LAT1-mediated efflux, but not influx of leucine. **a** LAT1/4F2hc-expressing oocytes (see Fig. 5) were washed and then exposed to buffer containing 100 µM [^{3}H]leucine (10 µCi/ml) for 15 min in the absence or presence of 1 mM FET. Cells were then washed in ice-cold buffer and lysed (n = 20–21). **b** LAT1/4F2hc-expressing oocytes were injected with 40 nl [^{3}H]leucine (10 mM, 100 µCi/ml) and immediately transferred to buffer containing either 1 mM FET, 1 mM Leu or no amino acid. The radioactivity in the supernatant was determined after 30 min and for each oocyte batch the mean values from oocytes incubated in leucine was set as 100 % (n = 13). **c** To decrease the intracellular amino acid concentration, LAT1-expressing oocytes were kept in buffer without any amino acid for 2 days, with buffer changes every 12 h. Oocytes were then injected with either 40 nl water containing 10 mM FET, or 10 mM Leu, or with water alone. Immediately after, the uptake of [^{3}H]leucine was measured as in **a** (n = 4–6). The radioactivity of lysates (**a**, **c**) and supernatants (**b**) was determined by scintillation counting. Values from oocytes, not overexpressing an exogenous transporter were subtracted. Columns represent mean ± S.E.M. Statistical analyses were performed using t test (**a**) or one-way ANOVA with Bonferroni's posttest (**b** and **c**). *Three stars* represent $p \leq 0.001$, no labeling corresponds to $p > 0.05$, compared to control conditions (no amino acids), respectively

However, FET still accumulated significantly. This can be explained by the still relatively high residual LAT1 expression in the knock-down cells that we found was comparable to LAT1 expression in human U373 MG glioblastoma cells (ATCC HTB-17, data not shown). Interestingly, high LAT1 expression has recently been reported in activated T lymphocytes, both, of human and murine origin (Hayashi et al. 2013; Sinclair et al. 2013). Together with our data, this suggests that FET may also accumulate in tumor-associated T cells. Regarding immune cells, FET accumulation has only been studied in a model of soft tissue infection so far, where macrophages and neutrophil granulocytes proved negative in this respect (Kaim et al. 2002).

Besides transporter number, the concentration of intracellular amino acids that serve as efflux substrates is an important determinant of LAT1 activity (Meier et al. 2002). As already discussed by Halvor Christensen, one of the pioneers of amino acid transport research, long before the molecular identification of system L transporters, the expression of amino acid transporters that install high intracellular concentrations of such efflux substrates may be another important factor for the accumulation of specific substrates by system L (Christensen 1990). Methionine is such an efflux substrate that can be concentrated within cells for example by Na^+-dependent system A transporters. His group observed that efflux of methionine, but not phenylalanine, was reduced in response to inhibition of system L, suggesting methionine, but not phenylalanine, to be a good efflux substrate of system L (Christensen and Handlogten 1979). This is compelling in the context that a recent report describes accumulation of p-(2-[^{18}F]fluoroethyl)-L-phenylalanine (FEP) and p-(3-[^{18}F]fluoropropyl)-L-phenylalanine (FPP) by LAT1 (Wang et al. 2011). It would be interesting to see if FEP and FPP are also recognized with different affinities at the intra- and extracellular binding pocket of LAT1. However, 3-[^{125}I]iodo-α-methyl-L-tyrosine (IMT) seems to be less well retained in LAT1-expressing cells and has been shown to be an efflux substrate for LAT1 (Shikano et al. 2003). Recoginition of a given derivative at the intracellular binding site does hence not only depend on the parent amino acid, but also on the substitution made.

LAT1 is also expressed in endothelial cells of the blood–brain barrier and supposed to mediate the transfer of peripheral FET into the cerebrospinal fluid (Lyck et al. 2009). Our data suggest LAT1 to be important for FET uptake from the blood into the endothelial cells, but not for its efflux into the CNS. Other transporter(s) must thus mediate the latter. In contrast to human glioblastoma cells, FET effluxes quickly from porcine SW 707 colon carcinoma cells, further supporting the existence of efflux transporter(s).

In rat F98 cells, $ATB^{0,+}$ and LAT2 were proposed to mediate FET transport in a Na^+-dependent (70 %) and

independent manner (30 %), respectively (Langen et al. 2003; Pauleit et al. 2005). Our trans-stimulation experiments in *X. laevis* oocytes revealed that FET is in fact also a substrate for LAT2 (data not shown). However, $ATB^{0,+}$-mediated leucine transport in *X. laevis* oocytes was not inhibited by FET. The Na^+-dependence of FET transport in F98 cells could probably also be explained by accumulation of intracellular trans-substrate for LAT2 (such as methionine) by Na^+-dependent transporters as discussed above. If FET uptake or accumulation in a given cell type is Na^+-dependent could best be answered using tritiated FET that is, however, not commercially available. In experiments with *X. laevis* oocytes, also y^+LAT2- and $b^{0,+}$AT-mediated leucine efflux was trans-stimulated by extracellular FET, demonstrating that FET is at least an uptake substrate for these transporters (data not shown). In contrast, y^+LAT1 did not recognize FET as a substrate.

Conclusion

Our data demonstrate that in human 229 glioblastoma cells, the Na^+-independent transporter LAT1 is responsible for intracellular accumulation of FET. This seems to be due to high LAT1 expression combined with asymmetric recognition of the amino acid derivative at the two sides of the membrane. A high concentration of LAT1 efflux substrates and the lack of an efflux transporter may further support the accumulation of FET in these tumor cells. In the larger picture, low intracellular affinity, respectively, asymmetry in substrate recognition may probably be exploited in the future for tumor-specific drug delivery and specific targeting in general. This may also apply substrates of other transporters.

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Conflict of interest The authors declare that they have no conflict of interest.

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