Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1664 (2004) 206–216

BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

L-Leucine transport in human breast cancer cells (MCF-7 and MDA-MB-231): kinetics, regulation by estrogen and molecular identity of the transporter

D.B. Shennan*, J. Thomson, I.F. Gow, M.T. Travers, M.C. Barber

Hannah Research Institute, Ayr, Scotland, KA6 5HL, UK

Received 2 March 2004; received in revised form 25 May 2004; accepted 28 May 2004
Available online 22 June 2004

Abstract

The transport of L-leucine by two human breast cancer cell lines has been examined. L-Leucine uptake by MDA-MB-231 and MCF-7 cells was via a BCH-sensitive, Na^+-independent pathway. L-Leucine uptake by both cell lines was inhibited by L-alanine, D-leucine and to a lesser extent by L-lysine but not by L-proline. Estrogen (17β-estradiol) stimulated L-leucine uptake by MCF-7 but not by MDA-MB-231 cells. L-Leucine efflux from MDA-MB-231 and MCF-7 cells was *trans*-stimulated by BCH in a dose-dependent fashion. The effect of external BCH on L-leucine efflux from both cell types was almost abolished by reducing the temperature from 37 to 4 °C. There was, however, a significant efflux of L-leucine under zero-*trans* conditions which was also temperature-sensitive. L-Glutamine, L-leucine, D-leucine, L-alanine, AIB and L-lysine all *trans*-stimulated L-leucine release from MDA-MB-231 and MCF-7 cells. In contrast, D-alanine and L-proline had little or no effect. The anti-cancer agent melphalan inhibited L-leucine uptake by MDA-MB-231 cells but had no effect on L-leucine efflux. Quantitative real-time PCR revealed that LAT1 mRNA was approximately 200 times more abundant than LAT2 mRNA in MCF-7 cells and confirmed that MDA-MB-231 cells express LAT1 but not LAT2 mRNA. LAT1 mRNA levels were higher in MCF-7 cells than in MDA-MB-231 cells. Furthermore, LAT1 mRNA was more abundant than CD98hc mRNA in both MDA-MB-231 and MCF-7 cells. The results suggest that system L is the major transporter for L-leucine in both MDA-MB-231 and MCF-7 cells. It is possible that LAT1 may be the major molecular correlate of system L in both cell types. However, not all of the properties of system L reflected those of LAT1/LAT2/CD98hc.

Keywords: L-Leucine; Cancer cell; Estrogen

1. Introduction

System L is a mechanism which transports neutral amino acids across the plasma membrane. It operates in a manner which does not require Na^+ and is inhibited by BCH (2-amino-2-norbornane carboxylic acid) (e.g. see Refs. [1,2]). As well as being involved in amino acid transport, it is becoming apparent that system L mediates the transport of the thyroid hormone T_3 and could also be involved in drug and trace element metabolism [3–8]. Recent molecular studies have borne out earlier predictions, based on kinetic analysis, that system L is not a single transporter [9–15]. To date, three molecular correlates of system L have been identified. Two of them are heterodimers which consist of a light chain (LAT1 or LAT2) joined to a heavy chain (CD98hc) by a disulfide bond. LAT1 and LAT2 are predicted to have 12 transmembrane spanning domains whereas CD98 hc, a highly glycosylated protein, is believed to have one transmembrane helix. It is accepted that the light chains act as the catalytic subunit. LAT2 is generally believed to transport a wider range of neutral amino acids than LAT1 [2]. The third molecular correlate of system L (LAT3) is structurally distinct from LAT1 and LAT2, does not require CD98hc for its functional expression and appears to have a narrow substrate specificity [16].

It is apparent that system L expression may be related to tumour growth [17–19]. For example, LAT1(TA1/E16) is expressed in colorectal cancer but not in the normal colon [17]. In addition, LAT1, in cooperation with CD98hc, appears to promote the growth of rat liver tumour lesions

* Corresponding author. Tel.: +44-1292-476013; fax: +44-1292-674066.
E-mail address: shennand@hri.sari.ac.uk (D.B. Shennan).

0005-2736/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2004.05.008

[18,19]. It has also been reported that LAT1 is highly expressed in a variety of human tumour cell lines [20]. In this connection, we have recently shown that two human breast cancer cell lines, namely MDA-MB-231 and MCF-7, respectively estrogen receptor-negative and-positive cells, express system L at the functional and molecular level [21]. MDA-MB-231 and MCF-7 cells exhibited Na^+-independent, BCH-sensitive α-aminoisobutyric acid transport. Furthermore, MCF-7 cells expressed LAT1, LAT2 and CD98hc mRNA whereas MDA-MB-231 cells expressed only LAT1 and CD98hc mRNA. An unusual finding was that L-lysine, generally regarded not to be a substrate, interacted with system L in both cell lines, albeit with low affinity.

System L in human breast cancer cells could be an important diagnostic tool and/or therapeutic target. For example, LAT1 (E16) is expressed in primary breast cancer tissue [17] and thus could play an important role in tumour cell proliferation by providing certain essential neutral amino acids. There is also good evidence that melphalan, an alkylating agent used in the treatment of breast cancer, gains access to MCF-7 cells via system L [22]. The present study was undertaken to confirm and extend our recent findings relating to the expression and activity of system L in cultured human breast cancer cells. In this paper we have examined the kinetics, regulation and substrate specificity of L-leucine transport by MDA-MB-231 and MCF-7 cells to see if the properties of system L correlate with those of LAT1/LAT2/CD98hc. In this connection, we have examined the relative expression of LAT1, LAT2 and CD98hc in the two cell lines using real-time quantitative PCR. In addition, we have attempted to establish if system L in human breast cancer cells can function as a uniporter as well as an exchange mechanism as the mode of operation is important regarding the relationship between system L and other amino acid transporters [23].

2. Methods

2.1. Cell culture

MCF-7 and MDA-MB-231 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 μg/ml). Cells were seeded in 35-mm culture wells containing 2 ml of culture DMEM medium (with supplements) at a density of $0.3–1.0 \times 10^6$ cells per well. The cells were cultured for 1–3 days at 37 °C in a gas phase of air with 5% CO_2 and were used at 60–90% confluency.

2.2. Culture of MCF-7 and MDA-MB-231 cells in the presence of 17β-estradiol

MCF-7 and MDA-MB-231 cells were cultured in phenol red-free DMEM supplemented with L-glutamine (2 mM), charcoal-stripped fetal bovine serum (10%), penicillin (50 IU/ml) and streptomycin (50 μg/ml). Cells were initially cultured in 75-cm^2 flasks containing 12 ml of culture medium for 3 days. Following this the cells were harvested using trypsin and seeded in 35-mm culture wells at a density of 5×10^5 cells per well and cultured for a further 48 h in phenol red-free DMEM containing the supplements described above. The culture medium was supplemented with 17β-estradiol (prepared in ethanol) to give a final concentration of 10 nM. Control culture wells were supplemented with ethanol alone (1μl/ml).

2.3. Measurement of L-leucine and L-alanine uptake

The uptake of L-leucine and L-alanine by MDA-MBA-231 and MCF-7 cells was assayed respectively using [^{3}H]leucine and [^{3}H]alanine as tracer. The culture medium was removed and the cells were washed three times with 3 ml of a buffer containing (mM) 0.01 L-leucine or L-alanine, 135 NaCl or choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4 and were preincubated for 5 min at 37 °C in 2 ml of an appropriate buffer (see figure legends for precise details). L-Leucine and L-alanine uptake experiments were started by adding 1 ml of a buffer similar in composition to that just described except that it also contained 0.4 μCi [^{14}C]sucrose together with either 1.0 μCi [^{3}H]leucine or 1.0μCi [^{3}H]alanine. At a pre-determined time, the incubation buffer containing the radioisotopes was removed and the cells were washed four times with 3 ml of an ice-cold buffer containing (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. Following the washing procedure, the cells were lysed by incubating in 1 ml of distilled water for at least 3 h, during which time the lysate was frequently agitated. A portion of the lysate (0.5 ml) was mixed with 0.5 ml of 10% trichloroacetic acid (TCA) and left to stand for at least 15 min. The mixture was centrifuged for 3 min at 12,000 × *g* and 0.5 ml of the supernatant was prepared for counting by adding 10 ml of UltimaGold liquid scintillation fluid. Samples of each lysate (0.1 ml) were taken to determine the protein content using the Lowry method. The specific activity of the isotopes in the incubation medium was determined by counting the amount of radioactivity associated with 10 μl of buffer. L-Leucine and L-alanine uptake by MCF-7 and MDA-MB-231 cells was calculated according to Eq. (1):

$$[\text{amino acid}]_c = ([\text{amino acid}]_t - (F[\text{amino acid}]_m))/P \quad (1)$$

where $[\text{amino acid}]_c$ is the amount of L-leucine or L-alanine in the cells (pmol/mg protein), $[\text{amino acid}]_t$ is the total amount of L-leucine or L-alanine in the lysate (pmol), $[\text{amino acid}]_m$ is the amount of L-leucine or L-alanine in the incubation medium (pmol), *F* is the ratio of radiolabelled sucrose in the lysate to that in the incubation medium and *P* is the amount of protein in the lysate (mg).

2.4. Measurement of amino acid release

The efflux of L-leucine from MCF-7 and MD-MB-231 cells was measured using [^{3}H] leucine or [^{14}C] leucine as tracer. The culture medium was removed and the cells were washed three times with 2 ml of a buffer containing (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. The cells were loaded with radiolabelled L-leucine over a period of 10 min by incubating in 2 ml of a buffer similar in composition to that just described except that it also contained 1.0 μCi/ml [^{3}H] leucine or 0.25 μCi/ml [^{14}C] leucine. After loading, the cells were washed four times in rapid succession with a radioactive-free buffer similar in composition to that described above. The unidirectional efflux of radiolabelled leucine from the cultured cells was measured by the sequential addition and removal of 2 ml of buffer (see figure legends for precise details of composition) at 1-min intervals. At the end of the efflux wash-out period, the cells were incubated in 2 ml of 10% TCA for at least 3 h. Following this incubation, the samples were centrifuged at 12,000 × *g* for 5 min. The resultant supernatant (1 ml) was prepared for counting by adding 10 ml of UltimaGold liquid scintillation fluid. The fractional release was calculated for each 1-min collection period. This was taken as the ratio of the amount of radioactivity lost from the cells per minute to the amount of radioactivity in the cells at the start of the collection period. The radioactivity associated with the cells at a given time was determined by adding the radioactivity left in the cells at the end of the incubation to the radioactivity lost by the cells in each of the collection periods in reverse order. Efflux time courses were also analysed by plotting ln (N_t/N_0) as a function of time where N_t represents the amount of radioactivity remaining in the cells at time t and N_0 is the amount of radioactivity associated with the cells at the start of the experiment. The negative slopes of the graphs provided a measure of the unidirectional efflux rate constants.

2.5. RNA isolation and quantitative RT-PCR

Total cellular RNA was isolated from the breast cancer cells using Triazol reagent (InVitrogen). RNA was suspended in water and the concentration determined by the A_{260}, where one unit of absorbance measured at 260 nm corresponded to 40 μg of RNA. The integrity of the RNA was checked by electrophoresis in a 1.2% agarose gel containing 2.2 M formaldehyde and 0.5 μg/ml of ethidium bromide [24].

PCR primers for LAT1, LAT2 and CD98hc were designed from the sequences available in GenBank (accession numbers: LAT1,AF104032; LAT2,AF171669; CD98(4F2hc),AH001404). Primer pairs were designed to different exons to prevent the amplification of any contaminating DNA. The primer pairs used in the PCRs were as follows: LAT1, forward 5′ GAGGCCGTGGCCGTGGACTTCGGGAACTAT 3′ and reverse 5′ GGCGATCAGGAAGAGGCAGGCCAGGATGAA 3′; LAT2, forward 5′ AGCCTGATATCCCCGCCCCATCAAGATCA 3′ and reverse 5′ TTGGCGGGACCAAGGCAGGGAGGTAGGATA 3′; CD98hc, forward 5′ CTGGCGGGTCTGAAGGGGCGTCTCGATTAC 3′ and reverse 5′ TCACCCCCGTAGTTGGGAGTAAGGTCCAGA 3′. The expected sizes of the PCR products were 420 bp for LAT1, 422 bp for LAT2 and 224 bp for CD98hc. Confirmation that the RT-PCR products were those expected was done by sequencing or digestion with specific restriction endonucleases [21].

cDNAs were prepared from three individual preparations of MCF-7 and MDA-MB-231 cells. Aliquots (1 μg) of total RNA were reverse transcribed using MMLV reverse transcriptase (Promega) and random hexamers (Pharmacia). Quantitative real-time PCR was performed using Lightcycler SYBR-green technology (Roche) using cDNA equivalent to 40 ng of total RNA. The data were collected at a temperature just below the start of the melting curve. The amount of specific sequence in the input DNA was then determined by reference to a dilution series of a plasmid pCD98L1L2 (pBluescriptII SK+ into which the PCR products of CD98, LAT1, and LAT2 were cloned) performed at the same time in duplicate.

3. Materials

L-[4,5-^{3}H]Leucine (157 Ci/mmol), L-[U-^{14}C]leucine (306 mCi/mmol), L-[2,3]alanine (52 Ci/mmol) and [U-^{14}C]sucrose (643 mCi/mmol) were purchased from Amersham plc, Bucks, UK. All other chemicals, unless otherwise indicated, were obtained from Sigma, Poole, Dorset, UK.

4. Statistics

Differences were assessed using Student's paired or unpaired *t*-test as appropriate and were considered significant when $P < 0.05$.

5. Results

5.1. L-Leucine uptake by MDA-MB-231 and MCF-7 cells

Preliminary experiments revealed that the time course of L-leucine uptake, measured under sodium-free conditions, by MDA-MB-231 and MCF-7 cells was linear up to 1 min (results not shown). Therefore, in subsequent experiments, L-leucine uptake was assayed after 30 s of incubation. We examined the effect of BCH on L-leucine uptake in the absence and presence of extracellular Na^+. Fig. 1 shows that L-leucine influx into MDA-MB-231 and MCF-7 cells was not affected by replacing extracellular sodium with choline. Fig. 1 also shows that L-leucine uptake into both cell types

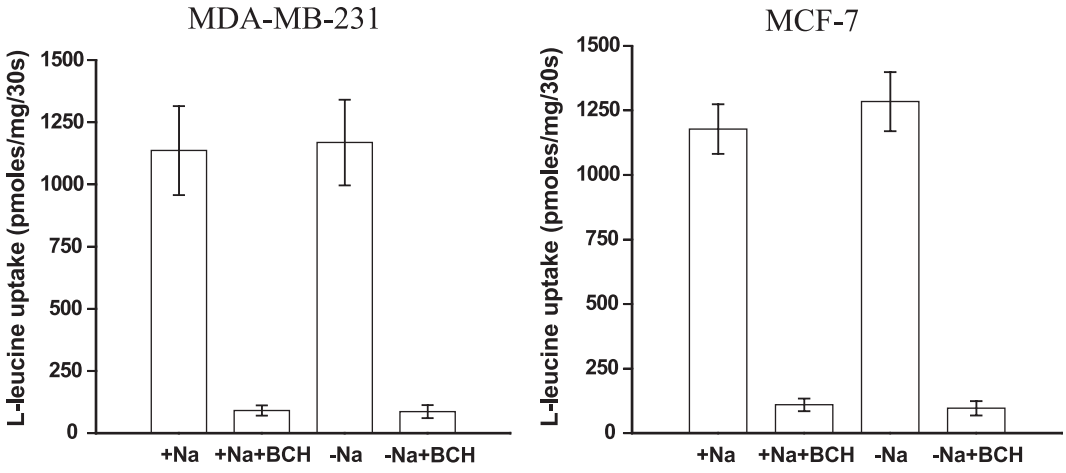

Fig. 1. The effect of BCH in the presence and absence of extracellular Na^+ on L-leucine uptake by MDA-MB-231 and MCF-7 cells. The incubation medium contained (mM) 0.01 L-leucine, 135 choline-Cl or NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. BCH, when required, was used at a concentration of 10 mM. Uptake was assayed at 37 °C after 30 s of incubation. The results shown are the means ± S.E. of four to five experiments, respectively, when MDA-MB-231 and MCF-7 cells were used.

was markedly inhibited (>90%) by BCH at a concentration of 10 mM. It is evident that the effect of BCH on L-leucine uptake was not affected by replacing extracellular Na^+ with choline. It appears, therefore, that the vast majority of L-leucine uptake into the two human breast cancer cell lines is via system L. In contrast, L-alanine uptake by both cell lines was significantly reduced by substituting extracellular Na^+ with choline. L-Alanine (10 μM) uptake by MDA-MB-231 cells in the presence and absence of extracellular Na^+ (choline replacement) was, respectively, 545.8 ± 84.4 and 36.2 ± 15.0 pmol/mg protein/30 s (± S.E.; $n=3$). L-Alanine uptake by MCF-7 cells in the presence and absence of extracellular Na^+ was, respectively, 476.2 ± 88.6 and 23.6 ± 8.7 pmol/mg protein/30 s (± S.E.; $n=3$).

We tested the effect of a variety of external amino acids on L-leucine influx into MDA-MB-231 and MCF-7 cells. The amino acids we examined were L-proline, L-alanine, L-lysine, D-leucine and unlabelled L-leucine. Each amino acid was added to the incubation medium to give a final concentration of 10 mM. L-Leucine uptake (10 μM) was measured under Na^+-free conditions. L-Leucine uptake into MDA-MB-231 cells was inhibited by unlabelled L-leucine (96.3 ± 0.6%; $P<0.001$), D-leucine (96.8 ± 0.5%; $P<0.01$), L-alanine (91.7 ± 1.7%; $P<0.001$) and L-lysine (65.7 ± 6.9%; $P<0.01$). On the other hand, L-proline was without effect. Similarly, L-leucine uptake by MCF-7 cells was inhibited by unlabelled L-leucine (96.8 ± 0.5%; $P<0.01$), D-leucine (92.7 ± 2.3%; $P<0.05$), L-alanine (76.4 ± 3.0%; $P<0.002$) and L-lysine (34.8 ± 7.7%; $P<0.02$) but not by L-proline.

The kinetic parameters (i.e. K_m and V_{max}) of L-leucine influx into MDA-MB-231 and MCF-7 cells were determined by incubating cells in media containing L-leucine concentrations ranging from 10 μM to 2 mM. L-Leucine uptake by MDA-MB-231 cells could be described by a single-saturable curve (Fig. 2). The K_m and V_{max} were, respectively, 46 ± 14 μM and 6.66 ± 0.25 nmol/mg protein/30 s. Similarly, L-leucine uptake by MCF-7 cells could also be described by a single-saturable curve despite the fact that these cells express both LAT1 and LAT2 mRNA [21]. Thus, the K_m and V_{max} were, respectively, 179 ± 41 μM and 20.78 ± 0.52 nmol/mg protein/30 s. Both the K_m ($P<0.05$) and V_{max} ($P<0.001$) of L-leucine uptake were lower in MDA-MB-231 cells compared to the corresponding values

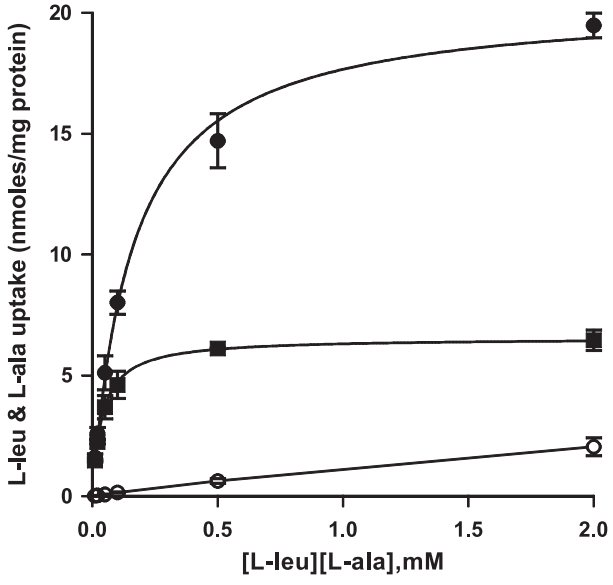

Fig. 2. The kinetics of L-leucine and L-alanine uptake by MCF-7 and MDA-MB-231 cells. The points denoted (●) and (○) respectively represent L-leucine and L-alanine uptake by MCF-7 cells and the points denoted (■) represent L-leucine uptake by MDA-MB-231 cells. The incubation medium contained (mM) 0.01–2 L-leucine or L-alanine, 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. Uptake was assayed at 37 °C after 30 s of incubation. Each point is the mean ± S.E. of four experiments for L-leucine uptake and three experiments for L-alanine uptake.

found with MCF-7 cells. Fig. 2 also illustrates L-alanine uptake by MCF-7 cells as a function of the extracellular L-alanine concentration. L-Alanine uptake was relatively low under Na^+-free conditions and operates with a K_m>2 mM.

Fig. 3 shows that L-leucine uptake by MDA-MB-231 and MCF-7 cells was inhibited by BCH in a dose-dependent manner. The IC_{50} value for BCH inhibition of L-leucine uptake into MDA-MB-231 and MCF-7 cells was, respectively, 88.9 ± 7.6 and 175.9 ± 28.3 μM. Fig. 3 also illustrates the effect of the amino acid-related compound melphalan on L-leucine uptake by MDA-MB-231 cells. Melphalan inhibited L-leucine influx in a dose-dependent manner: the IC_{50} was 82.5 ± 10.2 μM (± S.E., n = 3).

The effect of 17β-estradiol (10 nM) on the kinetics of L-leucine uptake via system L in MCF-7 cells was examined. In this set of experiments, cells were cultured in phenol red-free medium supplemented with charcoal-stripped fetal bovine serum. Culturing MCF-7 cells with 17β-estradiol increased the V_{max} of L-leucine uptake via system L without affecting the K_m (Table 1). Increasing the concentration of 17β-estradiol to 100 nM had no further effect on the V_{max} (results not shown). Furthermore, 17β-estradiol did not affect L-leucine uptake by MDA-MB-231 cells (Table 1).

5.2. L-Leucine efflux from MDA-MB-231 and MCF-7 cells

Preliminary experiments (Fig. 4, insets) established that the efflux of L-leucine from MDA-MB-231 and MCF-7 cells measured under Na^+-free conditions could be described by a monoexponential equation. The efflux rate constant and $T_{1/2}$ (calculated from ln 2/k) for [^{3}H]L-leucine efflux from MDA-MB-231 cells were, respectively, 0.100 ± 0.005 min^{-1} and 6.9 ± 0.4 min (± S.E., n = 3).

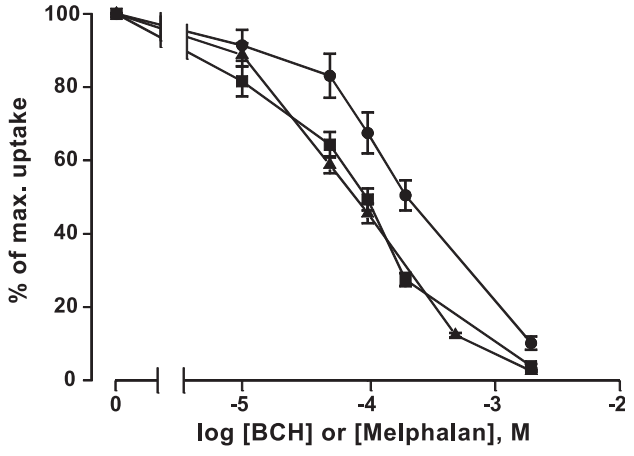

Fig. 3. Dose-response of BCH-inhibition of L-leucine uptake by MDA-MB-231 (■) and MCF-7 cells (●) and dose-response of melphalan inhibition of L-leucine uptake by MDA-MB-231 cells (▲). The incubation medium contained (mM) 0–2 BCH or 0–2 melphalan, 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. Uptake was assayed at 37 °C after 30 s of incubation. Each point is the mean ± S.E. of three to four experiments.

Table 1
The effect of 17β-estradiol on L-leucine uptake by MCF-7 and MDA-MB-231 cells

Cell line	17β-Estradiol	V_{max} (nmol/mg/30 s)	K_m (μM)
MCF-7	+	13.38 ± 0.99*	233.5 ± 37.2
	–	8.86 ± 0.47	187.1 ± 15.9
MDA-MB-231	+	9.72 ± 0.98	71.7 ± 8.7
	–	9.00 ± 1.02	60.5 ± 8.5

L-Leucine uptake was measured by MCF-7 and MDA-MB-231 cells which had been incubated for 48 h in the absence and presence of 17β-estradiol (10 nM). The incubation medium contained (mM) 0.01–2 L-leucine, 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 10 Tris–MOPS, pH 7.4 plus 1.0 μCi/ml [^{3}H]L-leucine and 0.4 μCi/ml [^{14}C]sucrose. Uptake was measured at 37 °C over 30 s. Values shown are the means ± S.E. of five and four experiments, respectively, when MCF-7 and MDA-MB-231 cells were used.

* P < 0.001.

The efflux rate constant and $T_{1/2}$ for [^{3}H]L-leucine efflux from MCF-7 cells were, respectively, 0.086 ± 0.001 min^{-1} and 8.1 ± 0.1 min (± S.E., n = 3). Importantly, [^{3}H]L-leucine and [^{14}C]L-leucine effluxes were almost identical (Fig. 4, insets). On the basis of cost, it was decided to routinely use [^{3}H]L-leucine as tracer in subsequent experiments. The effect of extracellular BCH, a paradigm substrate of system L, on L-leucine efflux from MDA-MB-231 and MCF-7 cells under Na^+-free conditions was examined. Fig. 4a shows the effect of BCH on L-leucine release from MDA-MB-231 cells. BCH *trans*-stimulated L-leucine efflux in a dose-dependent fashion. Thus, BCH at a concentration of 10, 50, 100, 200 and 2000 μM stimulated L-leucine efflux (basal-to-peak) by 130.0 ± 5.2%, 313.2 ± 16.8%, 492.6 ± 24.2%, 564.7 ± 11.9% and 664.3 ± 36.6%, respectively. To determine the apparent affinity constant for BCH at the external face of the carrier, we plotted V/V_o as a function of $((V/V_o) - 1)/[S]$ where V_o is L-leucine efflux in the absence of external BCH, V is L-leucine efflux in the presence of external BCH and $[S]$ is the BCH concentration in the medium. The apparent affinity constant was calculated from the slope of the plot. In applying this analysis we have made the following two assumptions: (1) the concentrations of BCH in the cytosol and that of L-leucine in the medium are zero at the start of the experiment; (2) the concentration of L-leucine in the cell is markedly less than its affinity for the internal face of the carrier [25]. The affinity constant for BCH, calculated in this manner, was 44.5 ± 3.6 μM (± S.E.; n = 3). Extracellular BCH also stimulated L-leucine efflux from MCF-7 cells in a dose-dependent fashion (Fig. 4b). Thus, external BCH at a concentration of 10, 50, 100 and 2000 μM increased L-leucine efflux (basal-to-peak) by 73.8 ± 11.9%, 258.9 ± 10.3%, 410.4 ± 13.4%, 505.2 ± 19.2% and 740.9 ±69.1%, respectively. The affinity constant for external BCH, calculated in a similar fashion to that described above, was 100.5 ± 20.5 μM(± S.E.; n = 4). Therefore, BCH stimulated L-leucine efflux from MCF-7 cells with lower affinity compared to MDA-MB-231 cells (P < 0.05).

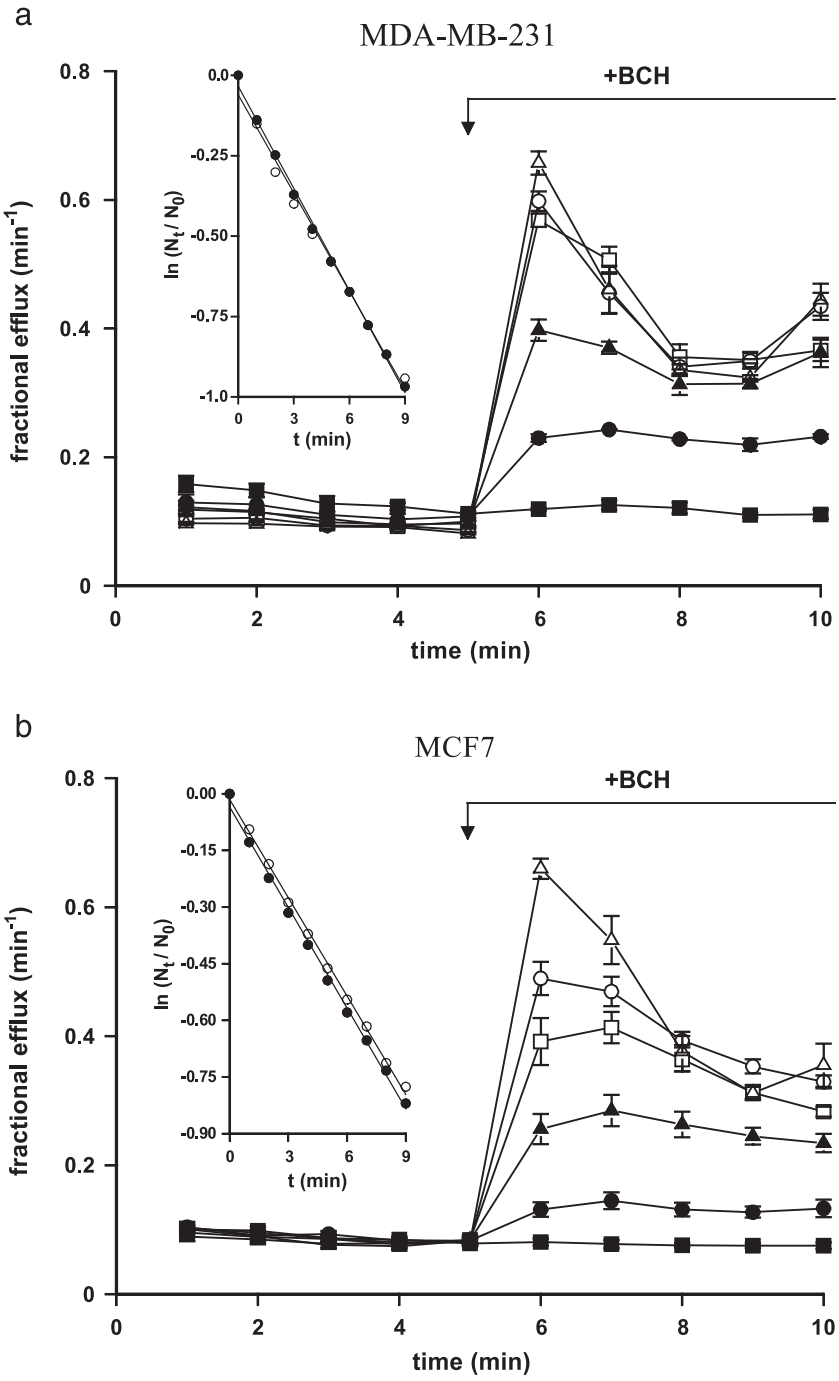

Fig. 4. The effect of extracellular BCH on L-leucine efflux from (a) MDA-MB-231 and (b) MCF-7 cells. The incubation medium contained (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. BCH was added to the incubation medium at a concentration of 1 μM (■), 10 μM (●), 50 μM (▲), 100 μM (□) 200 μM (○) and 2000 μM (△). Efflux was assayed at 37 °C. Each point is the mean ± S.E. of three and four experiments, respectively, when MDA-MB-231 and MCF-7 cells were used. Insets show the time course of [^{3}H]L-leucine (○) and [^{14}C]L-leucine (●) efflux from MDA-MB-231 and MCF-7 cells incubated in a medium containing (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. The plots are of ln (N_t/N_o) as a function of time where N_t represents the amount of radioactivity in the cells at time t and N_o represents the amount of radioactivity in the cells at the start of the incubation period.

Whilst it is evident from Fig. 4 that system L in MDA-MB-231 and MCF-7 cells can operate as an amino acid exchanger, it is apparent that L-leucine efflux persisted in the absence of external substrate. There is the possibility that amino acids released from the cells via alternative pathways could have acted to *trans*-stimulate L-leucine release. However, pre-incubating MDA-MB-231 cells for 1 h in an amino acid-free medium (with frequent change of the medium), to reduce the intracellular amino acid pool, did not diminish the fractional efflux of L-

leucine (0.0868 ± 0.0037 min^{-1}, $n=6$) measured under apparent zero-*trans* conditions. Therefore, the efflux of L-leucine measured from cells incubated in an amino acid-free buffer could possibly represent uncoupled transport via system L. To investigate this further, we examined the effect of temperature on L-leucine release from the two human breast cancer cell lines. Fig. 5 shows the effect of external BCH (2 mM) on leucine efflux from MDA-MB-231 cells measured at 37 and 4 °C. Reducing the temperature almost abolished the effect of extracellular BCH on L-leucine efflux. It is notable, however, that L-leucine efflux in the absence of BCH was also temperature-sensitive, suggesting that this moiety of efflux is carrier-mediated. Thus, L-leucine efflux from MDA-MB-231 cells was reduced from 0.0831 ± 0.0042 to 0.0236 ± 0.0027 min^{-1} ($\pm$ S.E.; $n=3$; $P<0.001$). Similarly, reducing the temperature of the incubation medium reduced L-leucine efflux from MCF-7 cells under zero-*trans* conditions from 0.0808 ± 0.0118 to 0.0303 ± 0.0046 min^{-1} ($\pm$ S.E.; $n=3$; $P<0.02$).

We examined the effect of a wide range of external amino acids (all tested at 2 mM) on L-leucine efflux from MDA-MB-231 cells in order to establish which amino acids are transported substrates. In this set of experiments, the efflux of L-leucine was initially measured from cells incubated in an amino-acid-free medium followed by a buffer containing a 'test' amino acid. The amino acids examined were L-leucine, D-leucine, L-glutamine, L-alanine, D-alanine, L-proline, L-lysine, L-arginine, L-glutamate and AIB. Table 2 shows that L-leucine, D-leucine, L-alanine and L-glutamine stimulated L-leucine release from MDA-MB-231 cells. AIB, L-lysine and D-alanine also *trans*-accelerated L-leucine efflux though less effectively than those just mentioned. In contrast, L-proline, L-arginine and L-glutamate had no significant effect on L-leucine efflux from MDA-MB-231 cells. The effect of amino acids on L-leucine efflux from MCF-7 cells showed a similar pattern (Table 3). Thus, L-glutamine, L-alanine and L-leucine and, to a lesser extent, AIB and L-lysine all *trans*-stimulated L-leucine efflux whereas L-proline and D-alanine were without effect.

The effect of external melphalan (2 mM) on L-leucine efflux from MDA-MB-231 cells was examined. Melphalan failed to trans-stimulate L-leucine release (Table 2). However, the effect of external BCH (0.5 mM) on L-leucine efflux from MDA-MB-231 cells was markedly reduced in the presence of external melphalan (2 mM) (results not shown).

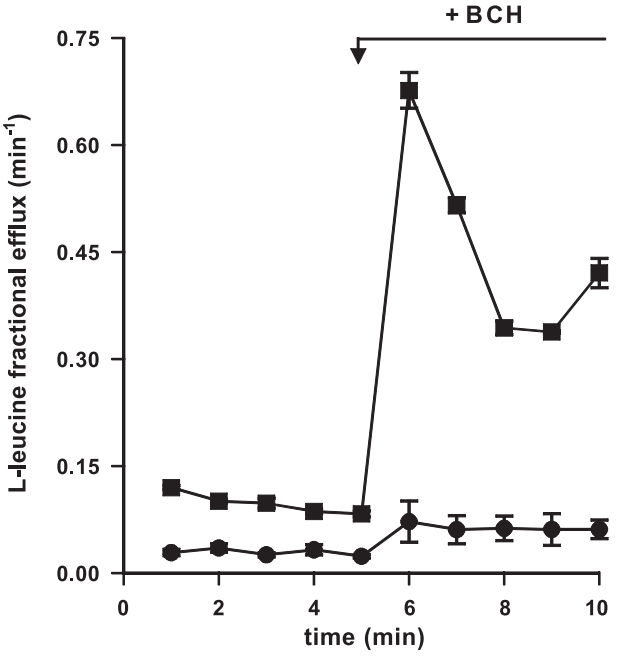

Fig. 5. L-Leucine efflux from MDA-MB-231 cells in the absence and MCF-7 cells in the absence and presence of external BCH measured at 37 °C (■) and 4 °C (●). The incubation medium contained (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. BCH, when required, was used at a concentration of 2 mM. Each point is the mean ± S.E. of three observations.

Table 2
The effect of external amino acids on L-leucine efflux from MDA-MB-231 cells

Fractional efflux (min^{-1}) × 10^{-2}						
Amino acid	n	− Amino acid	+ Amino acid	Difference	t	P
L-Leucine	3	7.37 ± 0.59	63.38 ± 4.49	56.01 ± 4.87	1	<0.01
D-Leucine	3	8.04 ± 0.79	69.90 ± 4.39	61.86 ± 3.79	1	<0.01
L-Alanine	4	8.98 ± 0.81	42.48 ± 3.21	33.50 ± 3.66	2	<0.01
D-Alanine	4	8.02 ± 0.61	12.77 ± 0.98	4.75 ± 0.46	5	<0.01
L-Glutamine	3	8.57 ± 0.65	50.00 ± 1.85	41.43 ± 2.17	1	<0.01
L-Lysine	5	10.28 ± 0.66	22.88 ± 3.26	12.60 ± 3.04	2	<0.02
L-Lysine (6 mM)	3	9.26 ± 0.64	31.69 ± 3.06	22.43 ± 2.67	2	<0.02
L-Arginine	3	11.69 ± 1.26	12.76 ± 1.61	–	–	NS
AIB	3	11.76 ± 1.71	34.91 ± 1.12	23.15 ± 2.53	3	<0.02
L-Proline	3	9.29 ± 1.04	10.64 ± 1.60	–	–	NS
L-Glutamate	4	13.32 ± 1.10	16.32 ± 1.19	–	–	NS
Melphalan	3	13.26 ± 1.51	11.84 ± 0.58	–	–	NS

Cells were incubated in a medium containing (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. Amino acids were used at a final concentration of 2 mM unless otherwise indicated. t represents the time taken for the fractional efflux to reach the peak value.

Table 3
The effect of external amino acids on L-leucine efflux from MCF-7 cells

Fractional efflux (min^{-1}) × 10^{-2}						
Amino acid	n	− Amino acid	+ Amino acid	Difference	t	P
L-Leucine	4	7.81 ± 0.53	66.62 ± 0.64	58.81 ± 0.98	1	<0.001
D-Leucine	3	7.91 ± 0.32	72.25 ± 0.16	64.34 ± 0.19	1	<0.001
L-Alanine	4	8.52 ± 0.95	27.44 ± 0.77	18.92 ± 0.25	3	<0.001
D-Alanine	3	7.97 ± 0.47	11.02 ± 0.11	–	–	NS
L-Glutamine	5	8.55 ± 0.49	44.80 ± 0.31	36.25 ± 0.71	2	<0.001
L-Lysine	4	8.24 ± 0.51	16.11 ± 0.26	7.87 ± 2.36	3	<0.05
AIB	3	8.77 ± 0.63	26.09 ± 0.19	17.32 ± 1.38	3	<0.01
L-Proline	3	7.64 ± 0.21	7.29 ± 0.61	–	–	NS

Cells were incubated in a buffer containing (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. Amino acids were used at a final concentration of 2 mM. t represents the time taken for the fractional efflux to reach a peak value.

Table 4
Quantitative expression of LAT1, LAT2 and CD98 mRNA in MDA-MB-231 and MCF-7 cells

	Transcripts per 40 ng of total RNA ($\times 10^3$)		
	LAT1	LAT2	CD98hc
MDA-MB-231	116.0 ± 7.2	ND	27.1 ± 1.9*
MCF-7	197.5 ± 20.8[a]	0.91 ± 0.10[†]	39.8 ± 7.3**

Values are means ± S.E. of three experiments. ND—not detected. CD98hc vs. LAT1 in both MCF-7 and MDA-MB-231 cells, * $P<0.02$, ** $P<0.01$; LAT1 vs. LAT2 in MCF-7 cells, [†] $P<0.02$; MCF-7 vs. MDA-MB-231 LAT1 levels [a] $P<0.05$.

5.3. Quantitative expression of LAT1, LAT2 and CD98hc

We examined the quantitative expression of LAT1, LAT2 and CD98hc in MDA-MB-231 and MCF-7 cells using real-time quantitative PCR (Table 4). The results confirm recent findings that MCF-7 cells express LAT1, LAT2 and CD98hc mRNA whereas MDA-MB-231 cells express LAT1 and CD98hc mRNA [21]. We now show that: (1) LAT1 mRNA is approximately 200 times more abundant than LAT2 mRNA in MCF-7 cells; (2) LAT1 mRNA is more abundant than CD98hc mRNA in both MCF-7 and MDA-MB-231 cells; (3) LAT1 mRNA is more abundant in MCF-7 cells than in MDA-MB-231 cells; and (4) CD98hc mRNA is more abundant than LAT2 mRNA in MCF-7 cells.

6. Discussion

6.1. L-Leucine is mediated by system L in human breast cancer cells

The results show that L-leucine uptake by MDA-MB-231 and MCF-7 cells is via a Na^+-independent, BCH-sensitive pathway, which is consistent with L-leucine uptake via system L. Indeed, it appears that system L may be the major, if not the sole, pathway for L-leucine uptake. This suggests that system L may play a crucial role in breast cancer cell growth by providing both cell types with essential neutral amino acids such as L-leucine. In this connection, a recent report from our laboratory also suggests that system L may be the major pathway for L-tryptophan uptake by MBA-MB-231 cells [26].

The present results are in accordance with our recent finding that AIB is transported via system L in both MDA-MB-231 and MCF-7 cells [21]. AIB was used to study system L in the two cell lines because we wanted to avoid the confounding issue of amino acid metabolism. Although AIB proved to be a useful tool for the initial characterization of system L [21], it has to be borne in mind that non-metabolizable amino acid analogues do have some limitations. In particular, the rate of AIB transport does not always approximate with that of naturally occurring amino acids. In this connection, the kinetic parameters of L-leucine and AIB uptake via system L are markedly different. Thus, the K_m of L-leucine uptake in MDA-MB-231 and MCF-7 cells is more than an order of magnitude smaller than that of AIB uptake. On the other hand, the V_{max} of L-leucine uptake by both cell lines is more than an order of magnitude greater than that of AIB uptake. Even though there was the possibility that L-leucine could have been subjected to metabolism, especially during the efflux experiments, there is evidence to suggest that L-leucine was a suitable substrate to use. First, the characteristics of Na^+-independent, BCH-sensitive L-leucine and AIB transport were similar. For example, those amino acids which were found to *trans*-accelerate AIB efflux also stimulated L-leucine efflux from both cell lines. Second, the effluxes of [^{14}C]L-leucine and [^{3}H]L-leucine were almost indistinguishable suggesting that metabolism may have been minimal.

6.2. Regulation of system L by estrogen

The results suggest that estrogen (17β-estradiol) regulates L-leucine uptake via system L in MCF-7 cells: the V_{max} of L-leucine uptake was increased whereas the K_m was unchanged. The effect appears to be specific given that 17β-estradiol had no effect on L-leucine uptake by estrogen receptor-negative MDA-MB-231 cells. In this connection, it has been reported that estrogen increases the expression of E16 (LAT1) in ZR75-1 cells (an estrogen receptor-positive human breast cancer cell line) [27]. It is tempting to suggest that estrogen may be a general regulator of amino acid transport in estrogen receptor-positive cells given that system A expression and activity in MCF-7 cells is also up-regulated by 17β-estradiol [28]. It is envisaged that estrogen could contribute to the proliferative capacity of estrogen receptor-positive breast cancer cells by increasing amino acid uptake via systems A and L.

The V_{max} of L-leucine uptake via system L was higher in MCF-7 cells than MDA-MB-231 cells when the cells are cultured in DMEM containing phenol red (see Fig. 2). However, this difference was abolished by culturing MCF-7 and MDA-MB-231 cells in phenol red-free DMEM supplemented with charcoal-stripped fetal bovine serum. This is consistent with the finding that estrogen regulates L-leucine transport via system L in MCF-7 cells.

6.3. Can system L activity in MDA-MB-231 and MCF-7 cells be ascribed to LAT1 and/or LAT2?

The data suggest that MDA-MB-231 cells express system L at the functional and molecular level. One important question that arises is: can the properties of system L in MDA-MB-231 cells be attributed to LAT1? L-Leucine transport in MDA-MB-231 cells shares many characteristics with amino acid transport via LAT1. For example, the K_m of L-leucine uptake is similar to that of L-leucine transport via LAT1 in T24 bladder cells [5]. The interaction of D-leucine with L-leucine transport is also reminiscent of LAT1. In

addition, the finding that amino acid efflux from MDA-MB-231 cells can be *trans*-accelerated is characteristic of LAT1. However, some of the properties of L-leucine transport in MDA-MB-231 cells are different from that of LAT1. In particular, we have confirmed the rather curious finding that L-lysine interacts with system L in MDA-MB-231 cells. The present results imply that L-lysine is actually a transported substrate. In contrast, LAT1 does not appear to accept L-lysine as substrate (e.g. see Ref. [9]). We must stress that the interaction between L-lysine and system L is one of low affinity. We also found that external L-alanine, which is considered not to be a substrate of LAT1, stimulated L-leucine efflux from MDA-MB-231 cells. However, L-alanine uptake (10 μM) by MDA-MB-231 cells under Na^+-free conditions was extremely low in comparison to L-leucine influx, suggesting that the former is a relatively poor (low affinity) substrate. Nevertheless, the results of the present study suggest that LAT1 in MDA-MB-231 human breast cancer cells can operate with a relatively wide substrate specificity. Alternatively, other molecular forms may contribute to system L activity in MDA-MB-231 cells. On the basis of kinetics and substrate specificity, we can rule out the recently discovered LAT3 molecular form of system L as being a major contributor to L-leucine uptake in MDA-MB-231 cells [16].

Attributing the characteristics of system L activity in MCF-7 cells to a particular molecular form is more difficult given that LAT1 and LAT2 are both expressed. However, the finding that LAT1 mRNA is approximately 200 times more abundant than LAT2 mRNA suggests that LAT1 may make a larger contribution to system L activity. The kinetics of L-alanine uptake by MCF-7 cells also suggests that the contribution of LAT2 to system L activity is relatively small. The ratio of L-leucine to L-alanine uptake (at a concentration of 0.1 mM) by MCF-7 cells is 51:1 whereas the ratio of L-leucine to L-alanine uptake by LAT2 expressed in *Xenopus* oocytes is approximately 2:1 [15].

6.4. Transport of melphalan by MDA-MB-231 cells

System L in MDA-MB-231 cells was inhibited by the anti-cancer agent melphalan. This is consistent with the notion that melphalan can gain entry into cells via system L [5,22]. However, we found that melphalan did not *trans*-stimulate L-leucine efflux from MDA-MB-231 cells, suggesting that it is transported slowly. Indeed, the rate of melphalan transport may be so low in comparison to amino acids that it acts more like a blocker than a transported substrate. It is predicted that melphalan at suitably high concentrations could inhibit the uptake of certain essential amino acids (e.g. L-leucine and L-tryptophan) into breast cancer cells. However, it must be borne in mind that firm conclusions regarding the effect of melphalan (and indeed BCH) on system L rely on the assumption that the effects of the compounds are relatively specific.

6.5. Can system L act as both an exchanger and a uniporter?

LAT1 operates as an amino acid exchange mechanism [9,29]. However, the mode of operation of LAT2 is a matter of controversy. On the one hand, it has been reported that LAT2 operates as an amino acid exchanger [12,15,29]. On the other hand, there is evidence to suggest that amino acid efflux via LAT2 is not dependent upon extracellular amino acids [13,30]. The operating mode of system L is important regarding its relationship with other amino acid transport mechanisms [23,29]. If, for instance, system L acts as a tightly coupled amino acid exchanger, it could not by itself accomplish net transport. Rather, it must function in parallel with other transport systems which have overlapping substrate specificity [23]. However, this need not be the case if system L allows some slippage. In this connection, we have confirmed that system L in MDA-MB-231 and MCF-7 cells is able to operate as an amino acid exchange mechanism. Thus, BCH stimulated L-leucine efflux from both human breast cancer cell lines with relatively high affinity. Other amino acids which *trans*-stimulated L-leucine efflux and which must be regarded as transported substrates include L-alanine, L-glutamine, AIB and D-leucine. However, system L in human breast cancer cells may also act as a uniporter. This notion is based on the finding that there is a significant amount of L-leucine efflux from MDA-MB-231 and MCF-7 cells under zero-*trans* conditions. This portion of L-leucine efflux can be reduced by lowering the temperature of the incubation medium, suggesting that it is carrier-mediated. In addition, it has recently been shown that there is a significant efflux of AIB from human breast cancer cell lines incubated in an amino acid-free buffer [21]. We feel that it is reasonable to suggest that this moiety of efflux may be uncoupled amino acid transport via system L. There are several lines of evidence that system L in other cell types is able to mediate net transport of amino acids. First, net (i.e. uncoupled) BCH transport occurs in human T-lymphocytes [31]. Second, despite the finding that lactating rat mammary tissue expresses system L at the molecular (LAT1 and LAT2) and functional level [32], amino acid efflux from mammary tissue explants was not trans-stimulated by system L substrates [33,34]. Third, Segawa et al. [13] and Liu et al. [30] have shown that LAT2 is capable of acting as a uniporter. Therefore, it appears that system L in some cells may not have to rely on other transporters to accomplish net transport.

6.6. Relative expression of LAT1, LAT2 and CD98 mRNA

The present study confirms that MCF-7 cells express LAT1, LAT2 and CD98hc mRNA but that MDA-MB-231 cells express only LAT1 and CD98hc mRNA. A new finding is that the abundance of LAT1 mRNA is markedly greater than that of CD98hc mRNA in both cell lines. The ratio of LAT1/CD98hc mRNA in MDA-MB-231 and MCF-

7 cells was, respectively, 4.3:1 and 4.9:1 In this connection Kim et al. [5] have reported that LAT1 mRNA is approximately 1.5 times higher than CD98hc mRNA in T24 human bladder carcinoma cells. It is interesting to note that the concentration of LAT1 mRNA found in T24 cells by Kim et al. [5] is comparable to that found by us in MDA-MB-231 and MCF-7 cells ($10.44 \pm 0.97 \times 10^{-18}$, $8.20 \pm 0.86 \times 10^{-18}$ and $4.82 \pm 0.29 \times 10^{-18}$ mol/μg of total RNA for T24, MCF-7 and MDA-MB-231 cells, respectively). The high ratio of LAT1/CD98 in the two mammary cancer cell lines appears to be unusual given that the expression of the heavy chain, but not the light chain, correlates with the induction of system L activity in human placental villous explants [35]. The significance of the LAT1/CD98hc mRNA ratio awaits the determination of relevant LAT1 and CD98 protein abundances and cellular location.

Acknowledgements

This study was funded by the Scottish Executive Environment and Rural Affairs Department.

References

[1] R. Deves, C.A.R. Boyd, Surface antigen CD98(4F2): not a single membrane protein, but a family of proteins with multiple functions, J. Membr. Biol. 173 (2000) 165–177.
[2] C.A. Wagner, F. Lang, S. Broer, Function and structure of heterodimeric amino acid transporters, Am. J. Physiol. 281 (2001) C1077–C1093.
[3] J.W. Ritchie, P.M. Taylor, Role of the system L permease LAT1 in amino acid and iodothyronine transport in placenta, Biochem. J. 356 (2001) 719–725.
[4] E.C. Friesema, R. Docter, E.P. Moerings, F. Verrey, E.P. Krenning, G. Hennemann, T.J. Visser, Thyroid hormone transport by the heterodimeric human system L amino acid transporter, Endocrinology 142 (2001) 4339–4348.
[5] D.K. Kim, Y. Kanai, H.W. Choi, S. Tangtrongsup, A. Chairoungdua, E. Babu, K. Tachampa, N. Anzai, Y. Iribe, H. Endou, Characterization of the system L amino acid transporter in T24 human bladder carcinoma cells, Biochim. Biophys. Acta 1565 (2002) 112–122.
[6] Y. Kanai, H.H. Endou, Heterodimeric amino acid transporters: molecular biology and pathological and pharmacological relevance, Curr. Drug Metab. 2 (2001) 339–354.
[7] H. Uchino, Y. Kanai, do K. Kim, M.F. Wempe, A. Chairoungdua, E. Morim, M.W. Anders, H. Endou, Transport of amino acid-related compounds mediated by L-type amino acid transporter 1 (LAT1): insights into the mechanism of substrate recognition, Mol. Pharmacol. 61 (2002) 729–737.
[8] T.A. Simmons-Willis, A.S. Koh, T.W. Clarkson, N. Ballatori, Transport of a neurotoxicant by molecular mimicry: the methylmercury–L-cysteine complex is a substrate for human LAT1 and LAT2 amino acid carriers, Biochem. J. 367 (2002) 239–246.
[9] Y. Kanai, H. Segawa, K. Miyamoto, H. Uchino, E. Takeda, H. Endou, Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98), J. Biol. Chem. 273 (1998) 23629–23632.
[10] L. Mastroberardino, B. Spindler, R. Pfeiffer, P.J. Skelly, J. Loffing, C.B. Shoemaker, F. Verrey, Amino-acid transport by heterodimers of 4F2hc/CD98 and members of a permease family, Nature 395 (1998) 288–291.
[11] P.D. Prasad, H. Wang, W. Huang, R. Kekuda, D.P. Rajan, F.H. Leibach, V. Ganapathy, Human LAT1, a subunit of system L amino acid transporter: molecular cloning and transport function, Biochem. Biophys. Res. Commun. 255 (1999) 283–288.
[12] M. Pineda, E. Fernandez, D. Torrents, R. Estavez, C. Lopez, M. Camps, J. Lloberas, A. Zorzano, M. Palacin, Identification of a membrane protein, LAT-2 that co-expresses with 4F2 heavy chain, an L-type amino transport activity with broad substrate specificity for small and large zwitterionic amino acids, J. Biol. Chem. 274 (1999) 19738–19744.
[13] H. Segawa, Y. Fukasawa, K. Miyamoto, E. Takeda, H. Endou, Y. Kanai, Identification and functional characterization of a Na^+-independent neutral amino acid transporter with broad substrate specificity, J. Biol. Chem. 274 (1999) 19745–19751.
[14] D.P. Rajan, R. Kekuda, W. Huang, L.D. Devoe, F.H. Leibach, P.D. Prasad, V. Ganapathy, Cloning and functional characterization of a Na^+-independent, broad-specific neutral amino acid transporter from mammalian intestine, Biochim. Biophys. Acta 1463 (2000) 6–14.
[15] G. Rossier, C. Meiers, C. Bauch, V. Summa, B. Sordat, F. Verrey, L.C. Kuhn, LAT2, a new basolateral 4F2hc/CD98-associated amino acid transporter of kidney and intestine, J. Biol. Chem. 274 (1999) 34948–34954.
[16] E. Babu, Y. Kanai, A. Chairoungdua, D.K. Kim, Y. Tribe, S. Tangtrongsup, P. Jutabha, Y. Li, N. Ahmed, S. Sakamoto, N. Anzai, S. Nagamori, H. Endou, Indentification of a novel system L amino acid transporter structurally distinct from heterodimeric amino acid transporters, J. Biol. Chem. 278 (2003) 43838–43845.
[17] D.A. Wolf, S. Wang, M.A. Panzica, N.H. Bassily, N.L. Thompson, Expression of a highly conserved oncofetal gene, TA1/E16, in human colon carcinoma and other primary cancers: homology to *Schistosoma mansoni* amino acid permease and *Caenorhabditis elegans* gene product, Cancer Res. 56 (1996) 5012–5022.
[18] S. Tamai, H. Masuda, Y. Ishii, S. Susuki, Y. Kanai, H. Endou, Expression of L-type amino acid transporter 1 in a rat model of liver metastasis: positive correlation with tumor size, Cancer Detec. Prev. 25 (2001) 439–445.
[19] H. Ohkame, H. Masuda, Y. Ishii, Y. Kanai, Expression of L-type amino acid transporter 1 (LAT1) and 4F2 heavy chain (4F2hc) in liver tumor lesions of rat models, J. Surg. Oncol. 78 (2001) 265–272.
[20] O. Yanagida, Y. Kanai, A. Chairoungdua, D.K. Kim, H. Segawa, T. Nii, S.H. Cha, H. Matsuo, J. Fukushima, Y. Fukasawa, Y. Tani, Y. Taketani, H. Uchino, J.Y. Kim, J. Inatomi, I. Okayasu, K. Miyamoto, E. Takeda, T. Goya, H. Endou, Human L-type amino acid transporter 1 (LAT1): characterization of a function and expression in tumor cell lines, Biochim. Biophys. Acta 1514 (2001) 291–302.
[21] D.B. Shennan, J. Thomson, M.C. Barber, M.T. Travers, Functional and molecular characteristics of system L in human breast cancer cells, Biochim. Biophys. Acta 1611 (2003) 81–90.
[22] J.A. Moscow, C.A. Swanson, K.H. Cowan, Decreased melphalan accumulation in a human breast cancer cell line selected for resistance to melphalan, Br. J. Cancer 68 (1993) 732–737.
[23] F. Verrey, System L: heteromeric exchangers of large, neutral amino acids involved in directional transport, Pflugers Arch. 445 (2003) 529–533.
[24] L.G. Davis, M.D. Dibner, J.F. Battey, in: L.G. Davis, M.D. Bibner, J.F. Battey (Eds.), Basic Methods in Molecular Biology, Elsevier, Amsterdam, 1986, pp. 143–146.
[25] R. Deves, R.M. Krupka, A simple experimental approach to the determination of carrier transport parameters for unlabeled substrate analogs, Biochim. Biophys. Acta 556 (1979) 524–532.
[26] M.T. Travers, I.F. Gow, M.C. Barber, J. Thomson, D.B. Shennan, Indoleamine 2,3-dioxygenase activity and L-tryptophan transport in human breast cancer cells, Biochim. Biophys. Acta 1661 (2004) 106–112.
[27] M. Soulez, M.G. Parker, Identification of novel oestrogen receptor

target genes in human ZR75-1 breast cancer cells by expression profiling, J. Mol. Endocrinol. 27 (2001) 259–274.
[28] H.K. Bhat, J.V. Vadgama, Role of estrogen receptor in the regulation of estrogen induced amino acid transport of system A in breast cancer and other receptor positive tumor cells, Int. J. Mol. Med. 9 (2002) 271–279.
[29] C. Meier, Z. Ristic, S. Klauser, F. Verrey, Activation of system L heterodimeric amino acid exchangers by intracellular substrates, EMBO J. 21 (2002) 580–589.
[30] X. Liu, L. Charrier, A. Gewirtz, S. Sitaraman, D. Merlin, CD98 and ICAM-1 regulate the activity of amino acid transporter LAT-2 in polarised intestinal epithelia, J. Biol. Chem. 278 (2003) 23672–23677.
[31] G.B. Segel, T.J. Woodlock, M.A. Lichtman, Trans-stimulation of L-system amino acid transport in normal and chronic leukemic human lymphocytes: phorbol ester restores function in CLL, J. Cell. Physiol. 134 (1988) 503–508.
[32] D.B. Shennan, D.T. Calvert, M.T. Travers, Y. Kudo, C.A.R. Boyd, A study of L-leucine, L-phenylalanine and L-alanine transport in the perfused rat mammary gland: possible involvement of LAT1 and LAT2, Biochim. Biophys. Acta 1564 (2002) 133–139.
[33] D.B. Shennan, M. Peaker, Transport of milk constituents by the mammary gland, Physiol. Rev. 80 (2000) 925–951.
[34] D.B. Shennan, J. Thomson, M.T. Travers, Y. Kudo, C.A.R. Boyd, Properties and regulation of system L in lactating mammary tissue, in: P. Poujeol, O. Petersen (Eds.), Proceedings 3rd Federation of European Physiological Societies Congress, Monduzzi Editore Int. Proc. Division, Italy, 2003, pp. 247–251.
[35] Y. Kudo, C.A.R. Boyd, Heterodimeric amino acid transporters: expression of heavy chains of CD98 correlates with induction of amino acid transport systems in human placental trophoblasts, J. Physiol. 523.1 (2000) 13–18.