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Functional and molecular characteristics of system L in human breast cancer cells

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Abstract

The functional and molecular properties of system L in human mammary cancer cells (MDA-MB-231 and MCF-7) have been examined. All transport experiments were conducted under Na^+-free conditions. α-Aminoisobutyric acid (AIB) uptake by MDA-MB-231 and MCF-7 cells was almost abolished by BCH (2-amino-2-norbornane-carboxylic acid). AIB uptake by MDA-MB-231 cells was also inhibited by L-alanine (83.6%), L-lysine (75.6%) but not by L-proline. Similarly, L-lysine and L-alanine, respectively, reduced AIB influx into MCF-7 cells by 45.3% and 63.7%. The K_m of AIB uptake into MDA-MB-231 and MCF-7 cells was, respectively, 1.6 and 8.8 mM, whereas the V_{max} was, respectively, 9.7 and 110.0 nmol/mg protein/10 min. AIB efflux from MDA-MB-231 and MCF-7 cells was *trans*-stimulated by BCH, L-glutamine, L-alanine, L-leucine, L-lysine and AIB (all at 2 mM). In contrast, L-glutamate, L-proline, L-arginine and MeAIB had no effect. The interaction between L-lysine and AIB efflux was one of low affinity. The fractional release of AIB from MDA-MB-231 cells was *trans*-accelerated by D-leucine and D-tryptophan but not by D-alanine. MDA-MB-231 and MCF-7 cells expressed LAT1 and CD98 mRNA. MCF-7 cells also expressed LAT2 mRNA. The results suggest that AIB transport in mammary cancer cells under Na^+-free conditions is predominantly via system L which acts as an exchange mechanism. The differences in the kinetics of AIB transport between MDA-MB-231 and MCF-7 cells may be due to the differential expression of LAT2.

Keywords: System L; Breast cancer; Human breast cancer; α-Aminoisobutyric acid

1. Introduction

System L is a Na^+-independent, BCH-sensitive amino acid transport system which accepts neutral amino acids as substrates. It became apparent from functional studies that system L was not a single transporter and recent molecular studies have shown that there are at least two isoforms [1–3]. Two proteins, termed LAT1 and LAT2, induce system L-like activity when co-expressed with CD98 (formerly referred to as 4F2hc) [4–9]. Thus, the L system carrier protein is a heterodimer consisting of a light chain (LAT1 or LAT2) and a heavy chain (CD98), which are joined by a disulfide bond. LAT1 and LAT2 differ from one another with respect to their tissue distribution, kinetics, substrate specificity and pH dependence [3]. LAT1 preferentially transports large neutral amino acids such as L-phenylalanine, L-tryptophan, L-leucine and L-tyrosine with high affinity and also transports certain amino acid D-isomers. The substrate specificity of LAT2 appears to be somewhat wider than LAT1 but the amino acids are transported with lower affinity. Indeed, a major distinguishing feature between LAT1 and LAT2 is that the latter transports L-alanine at physiological concentrations [8,9]. It appears that LAT1 acts as an exchange mechanism, thus, amino acid transport via LAT1 is readily stimulated by *trans*-amino acids [4,10]. There appears to be some controversy as to whether or not LAT2 acts as an exchange system. On the one hand Pineda et al. [6], Rossier et al. [9] and Meier et al. [10] have shown that amino acid efflux via LAT2 depends upon the presence of extracellular substrate. In contrast, Segawa et al. [7] reported that LAT2 could operate as a uniporter.

It has long been recognised that the transport of amino acids via system L is important for cellular nutrition [11]. However, it is becoming apparent that system L can also transport amino acid related compounds [12,13]. For example, several groups have reported that LAT1 is able to transport iodothyronine [14–16]. In addition, system L

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may participate in metal ion homeostasis given that LAT1 and LAT2 transports methylmercury complexed to L-cysteine [17].

It has also been shown that LAT1/CD98 is related to tumour growth which has given rise to the suggestion that LAT1 may be a useful therapeutic target [18,19]. In relation to this, it has been reported that a variety of human tumour cell lines express LAT1 [20]. In spite of the potentially interesting relationship between system L (LAT1) and tumour size, very little is known about the properties of amino acid transport in human mammary cancer cells. In view of this, we have examined the functional and molecular characteristics of system L in two human breast cancer cell lines, namely MDA-MB-231 and MCF-7 which are, respectively estrogen receptor-negative and -positive cells. The results show that both cell lines exhibit system L activity. However, the functional characteristics of system L in the two cell lines appear to be different from that reported elsewhere and the molecular distribution of LAT1 and LAT2 differs between MCF-7 and MDA-MB-231 cells.

2. Methods

2.1. Culture of MDA-MB-231 and MCF-7 cells

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

2.2. Amino acid uptake by MDA-MB-231 and MCF-7 cells

The unidirectional influx of α-aminoisobutyric acid (AIB) into MDA-MB-231 and MCF-7 cells was assayed using [^{3}H]AIB (ICN Biomedicals, specific activity 10 Ci/mmol) as tracer. The culture medium was removed and the cells were washed (× 3) with 3 ml of a buffer containing (mM) 0.01 AIB, 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. Following this, the cells were preincubated for 5 min at 37 °C in 3 ml of an appropriate buffer (see figure legends for precise details). AIB uptake experiments were initiated by aspirating the preincubation buffer and replacing it with 1 ml of a buffer containing (mM) 0.01 AIB, 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4 plus 3 μCi of [^{3}H]AIB and 0.4 μCi [^{14}C] sucrose (Amersham PLC, specific activity 615 mCi/mmol), together with other additions such as unlabelled amino acids. Radiolabelled sucrose was used to correct for [^{3}H]AIB associated with the extracellular compartment (i.e. to correct for incomplete washing of the cells). The specific activity of the isotopes in the incubation buffer was determined by counting the radioactivity associated with 10 μl of buffer. These samples were prepared for counting by adding 0.5 ml of distilled H_2O and 10 ml of UltimaGold liquid scintillation fluid. At a predetermined time, the incubation buffer was aspirated and the cells were washed four times with 3 ml of an ice-cold solution containing (mM) 135 choline-Cl, 5 KCl, 2 KCl, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. Following this, the cells were incubated in 1 ml of distilled H_2O for at least 3 h during which time the lysate was frequently agitated. A sample of the lysate (0.5 ml) was prepared for counting by adding 10 ml of UltimaGold liquid scintillation cocktail. Samples of the lysate (0.1 ml) were taken to determine the protein content using the Lowry assay. AIB uptake by the cells was calculated according to Eq. (1):

$$[\mathrm{AIB}]_c = ([\mathrm{AIB}]_t - (F[\mathrm{AIB}]_m))/P \quad (1)$$

where $[AIB]_c$ is the amount of AIB in the cells (pmol/mg protein), $[AIB]_t$ is the total amount of AIB in the lysate (pmol), $[AIB]_m$ is the amount of AIB 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).

2.3. Amino acid efflux from MDA-MB-231 and MCF-7 cells

The efflux of AIB from MDA-MB-231 and MCF-7 cells was assayed using [^{3}H]AIB as tracer. After removing the culture medium, the cells were washed (× 3) 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 AIB over a period of 60 min at 37 °C by incubating in 2 ml of a buffer similar in composition to that just described except that it also contained 1–2 μCi/ml of [^{3}H]AIB. After the loading period, the cells were washed four times in rapid succession with a radioactive-free buffer similar in composition to that described above. The efflux of radiolabelled AIB from the cells was measured by the sequential addition and removal of 2 ml of buffer (see figure legends for details of composition) at 1-min intervals. The first sample was discarded because it contained a significant amount of isotope derived from the extracellular compartment. At the end of the washout period, cells were incubated in 2 ml of distilled water for at least 3 h to determine the amount of radioactivity remaining in the cells. Samples were prepared for counting by adding 10 ml of UltimaGold liquid scintillation cocktail. The fractional release for each collection period was calculated according to Eq. (2):

$$\text{Fractional release } (\mathrm{min}^{-1}) = \delta X/(\delta t X_t) \quad (2)$$

where δX is the amount of radiolabelled AIB released from the cells in the time interval δt and X_t is the amount of isotope in the cells at the start of the sampling period.

2.4. RNA isolation and RT-PCR

Total cellular RNA was isolated from human placental tissue (following removal of the maternal decidua and chorionic plate) and cultured mammary cells using guanidinium thiocyanate and caesium chloride centrifugation [21]. RNA was resuspended in water and the concentration determined by the A_{260}, where one A_{260} corresponds 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 [22].

PCR primers for LAT1, LAT2 and CD98 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 subsequent PCRs were as follows: LAT1, forward 5′GAGGCCGTGGCCGTGGACTTCGGGAGGCCGTGGCCGTGGACTTCGGGAACTAT 3′and reverse 5′GGCGATCAGGAAGAGGCAGGCCAGGATGAA 3′; LAT2, forward 5′AGCCTGATATCCCCCGCCCCATCAAGATCA 3′and reverse 5′TTGGCGGGACCTTGGCGGGACCAAGGCAGGGAGGTAGGATA 3′; CD98, forward 5′CTGGCGGGTCTGAAGGGGCGTCTCTGGCGGGTCTGAAGGGGCGTCTCGATTAC 3′and reverse 5′ TCACCCCCGTAGTTGGGAGTAAGGTC-

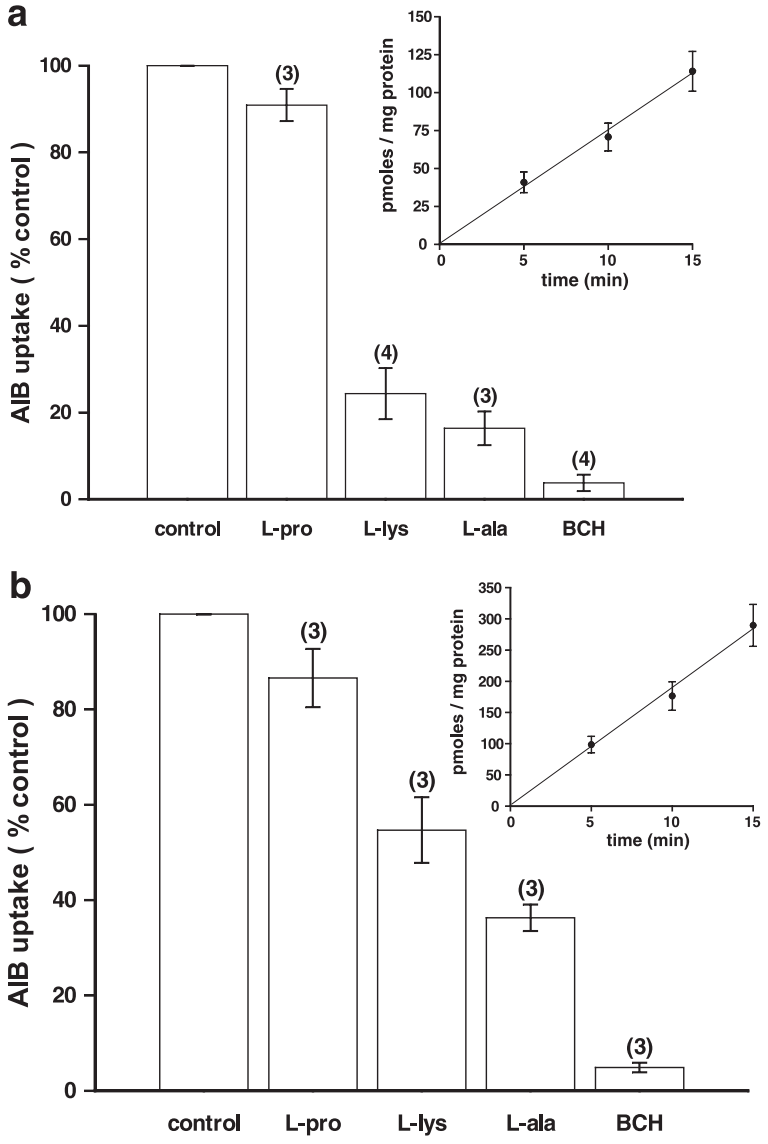

Fig. 1. The effect of external amino acids on AIB uptake by (a) MDA-MB-231 and (b) MCF-7 cells. Cells were incubated in a medium containing (mM) 0.01 AIB, 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4 plus 3 μCi [^{3}H]AIB, 0.4 μCi [^{14}C]sucrose ± amino acids (10 mM) as required. Uptake was assayed at 37 °C after 10 min of incubation. The numbers in parentheses represent the number of experiments performed with each amino acid. Insets show the time course of AIB uptake into MDA-MB-231 and MCF-7 cells ($n=3$; ± S.E.). The incubation medium was the same as that described above.

CAGA 3′. The expected sizes of the PCR products were 420 bp for LAT1, 442 bp for LAT2 and 224 bp for CD98.

cDNA was prepared from 1-μg samples of total RNA using M-MLV reverse transcriptase (Promega) and random hexamers (Pharmacia). The cDNA (0.05 μg equivalent to RNA) was PCR amplified using Taq polymerase (Thermo-Start Master Mix, ABgene) and 2 μM of each of the appropriate primer pair, in a total volume of 25 μl. Samples were initially denatured at 94 °C for 5 min, followed by 35 cycles of 92 °C for 30 s, 59.8 °C (for LAT1 and LAT2) or 60.3 °C (for CD98) for 30 s, and 72 °C for 30 s, with a final 5 min extension at 72 °C. Aliquots (5 μl) of the PCRs were then resolved on a 1% agarose/TBE gel and the products visualised by ethidium bromide staining. Confirmation that the RT-PCR products were those expected was done either by sequencing the products (LAT1 and LAT2) or by restriction digest analysis (CD98).

3. Statistics

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

4. Results

4.1. AIB uptake by MDA-MB-231 and MCF-7 cells

Preliminary experiments established that the uptake of AIB by both MDA-MB-231 and MCF-7 cells was linear for at least 15 min under Na^+-free conditions (Fig. 1a and b, insets). The rate of AIB uptake (10 μM) by MDA-MB-231 and MCF-7 cells was, respectively, 6.88 ± 0.89 and 19.11 ± 3.09 nmol/mg protein/min (± S.E., $n = 3$). The next step in the investigation was to test the effect of a variety of amino acids, added to the incubation medium, on AIB uptake by the cultured cells. In this set of experiments, the effect of each extracellular amino acid (10 mM) on AIB influx was assayed after 10 min of incubation. Fig. 1a shows that AIB uptake by MDA-MB-231 cells was markedly reduced by the system L inhibitor BCH (96.2 ± 1.9%; $P < 0.001$). Indeed, it appears that the BCH-sensitive pathway, and thus system L, is the major, if not only, pathway for AIB uptake by MDA-MB-231 cells under Na^+-free conditions. AIB uptake by MDA-MB-231 cells

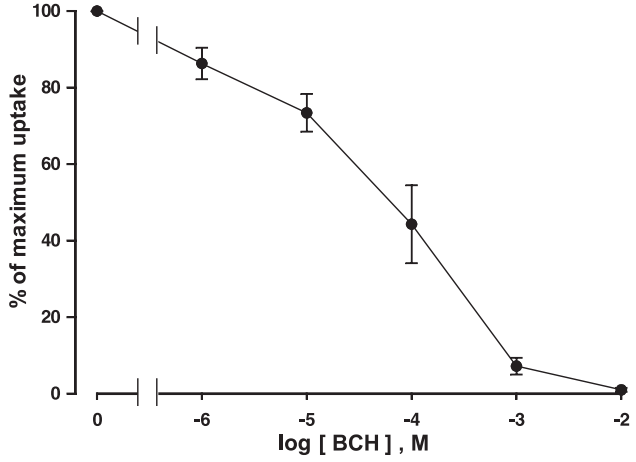

Fig. 2. Dose–response curve of BCH inhibition of AIB uptake by MDA-MB-231 cells. The incubation medium contained (mM) 0.001–10 BCH, 0.01 AIB, 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 10 Tris–MOPS, pH 7.4 plus 3 μCi [^{3}H]AIB and 0.4 μCi [^{14}C]sucrose. Uptake was assayed at 37 °C after 10 min of incubation. The results are mean ± S.E.; $n = 5$.

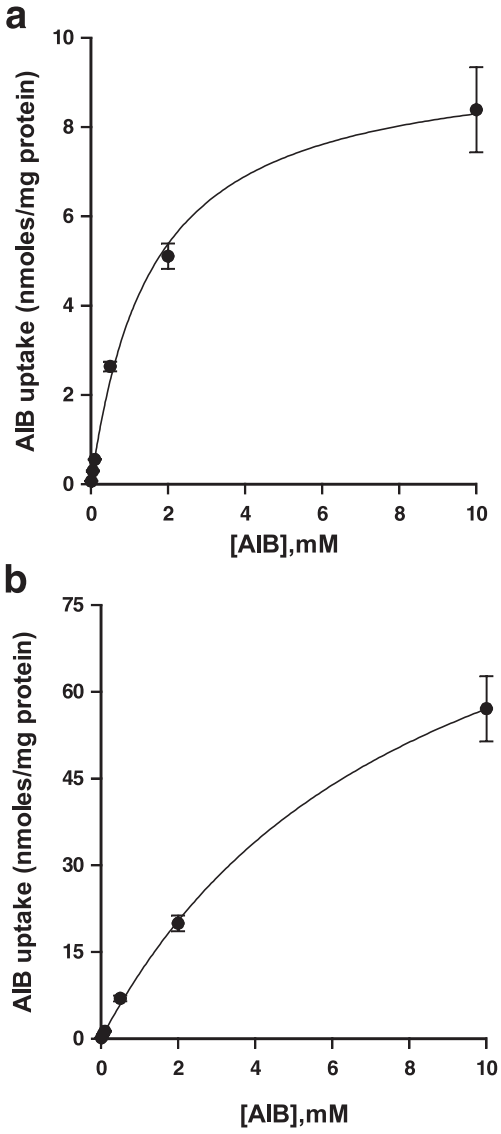

Fig. 3. The kinetics of AIB uptake by (a) MDA-MB-231 and (b) MCF-7 cells. The incubation medium contained (mM) 0.01–10 AIB, 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4 plus 3 μCi [^{3}H]AIB and 0.4 μCi [^{14}C]sucrose. Uptake was assayed at 37 °C after 10 min of incubation. Each point is the mean ± S.E. of four experiments.

was also inhibited by L-alanine (83.6 ± 3.9%; $P<0.01$) but not by L-proline. It is evident that the BCH-sensitive pathway for AIB uptake also accepts L-lysine as a substrate given that L-lysine inhibited AIB uptake by 75.6 ± 5.9% ($P<0.002$). Fig. 1b shows that the uptake of AIB by MCF-7 cells was also markedly inhibited by BCH (95.1 ± 1.0%; $P<0.01$). Again, it appears that the BCH-sensitive route, and thus system L, may be the only available pathway for AIB uptake by MCF-7 cells under Na^+-free conditions. The BCH-sensitive pathway for AIB uptake into MCF-7 cells was also inhibited by L-alanine (63.7 ± 2.8%; $P<0.001$) and L-lysine (45.3 ± 6.9%; $P<0.05$) but not by L-proline (Fig. 1b). Fig. 2 shows that BCH inhibited AIB uptake by MDA-MB-231 cells in a concentration-dependent manner. The IC_{50} was approximately 50 μM.

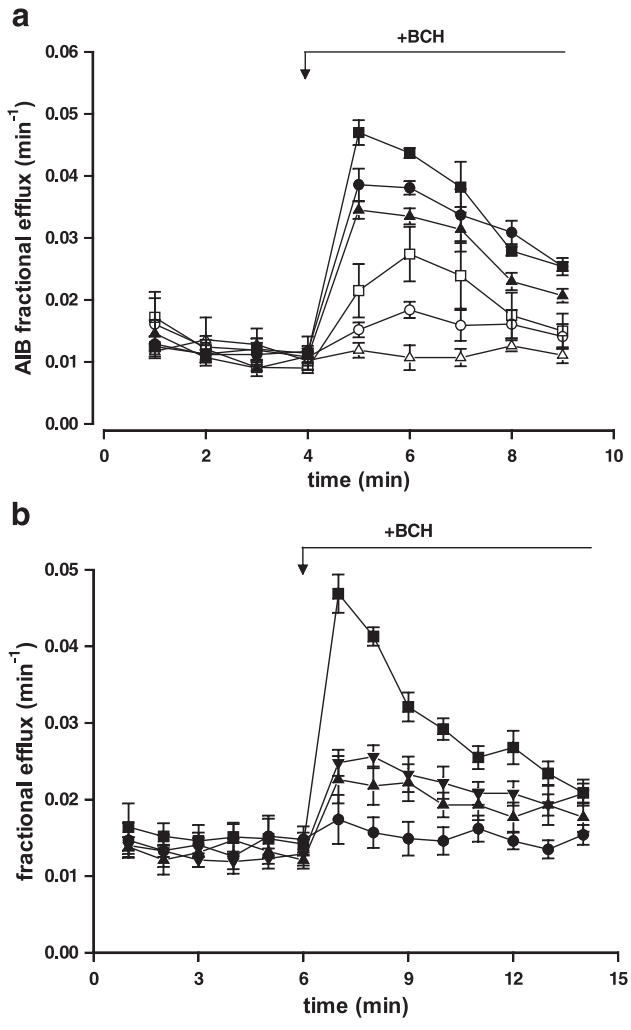

Fig. 4. The effect of external BCH on AIB efflux from (a) MDA-MB-231 and (b) MCF-7 cells. The incubation medium contained (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. When AIB efflux from MDA-MB-231 cells was examined, BCH was added to the incubation medium at a concentration of 1 μM (△), 10 μM (○), 50 μM (□), 75 μM (▲), 100 μM (●) and 2 mM (■). Each point is the mean ± S.E. of four experiments. When AIB efflux from MCF-7 cells was studied, BCH was added to the incubation medium at a concentration of 10 μM (●), 50 μM (▲), 100 μM (▼) and 2 mM (■). Each point is the mean ± S.E. of four experiments.

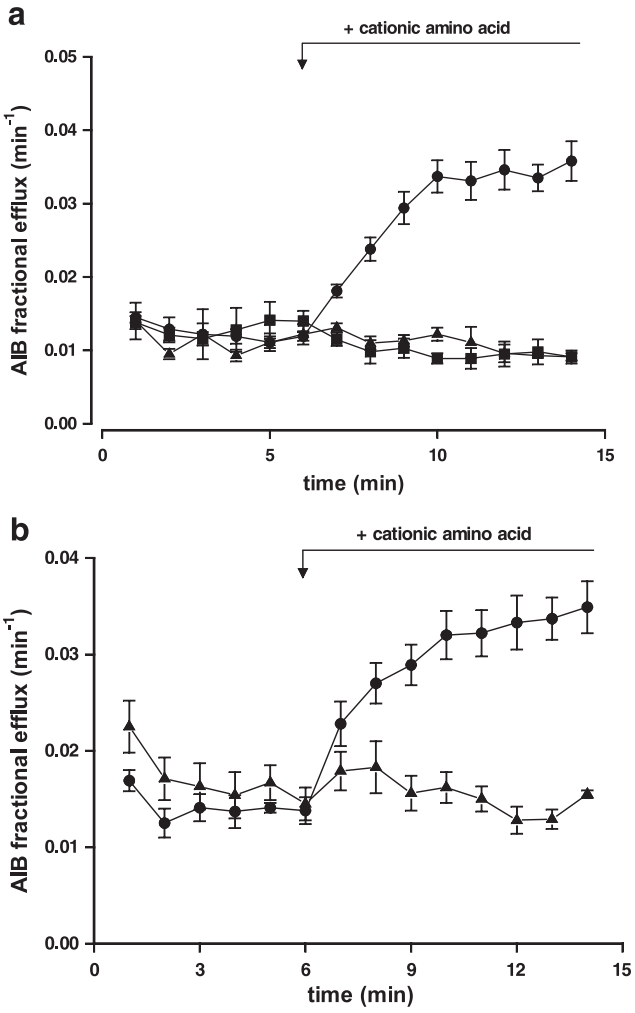

Fig. 5. (a) The effect of 2 mM L-lysine (●), 0.1 mM L-lysine (■) and 2 mM L-arginine (▲) on AIB efflux from MDA-MB-231. The incubation medium contained (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. Each point is the mean ± S.E. of ten, five and four experiments when the effects of 2 mM L-lysine, 0.1 mM L-lysine, and 2mM L-arginine were examined, respectively. (b) The effect of 2 mM L-lysine (●) and L-arginine (▲) on AIB efflux from MCF-7 cells. The incubation medium was the same as that described above. Each point is the mean ± S.E. of four and three experiments, respectively, when L-lysine and L-arginine was used.

AIB uptake by MDA-MB-231 and MCF-7 cells followed Michaelis–Menton kinetics (Fig. 3a and b). We are aware, however, that the kinetic data could be described by more complex models. The kinetic constants were lower in MDA-MB-231 cells compared with the values obtained with MCF-7 cells. The K_m of AIB uptake into MDA-MB-231 and MCF-7 cells was, respectively, 1.64 ± 0.36 and 8.81 ± 2.16 mM ($n=4$; ± S.E.; $P<0.02$). The V_{max} of AIB influx into MDA-MB-231 and MCF-7 cells was,

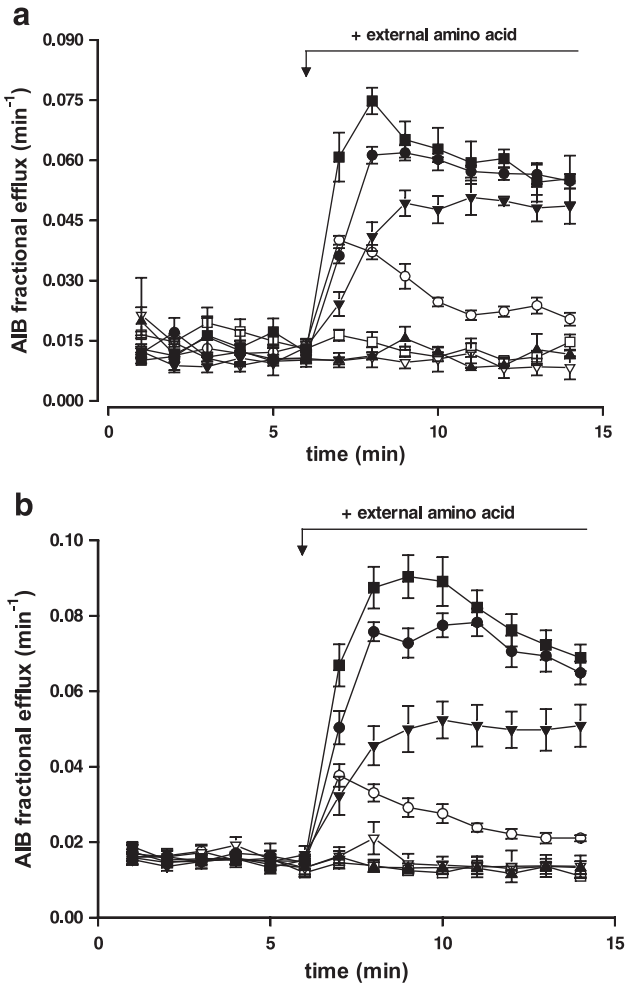

Fig. 6. The effect of external L-glutamine (■), L-alanine (●), L-leucine (○), L-proline (▲), L-glutamate (□), AIB (▼) and MeAIB (▽) on the efflux of AIB from (a) MDA-MB-231 and (b) MCF-7 cells. The incubation medium contained (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. External amino acids were added to the incubation medium to give a final concentration of 2 mM. Each point represents the mean ± S.E. of between three and six observations.

respectively, 9.79 ± 1.40 and 110.02 ± 21.72 nmol/mg protein ($n=4$; ± S.E.; $P<0.01$).

4.2. AIB efflux from MDA-MB-231 and MCF-7 cells

4.2.1. BCH trans-stimulates AIB efflux

There is good evidence that system L acts as an amino acid exchange mechanism (e.g. see Ref. [10]). To test whether or not system L in mammary tumour cells is an exchange mechanism, we examined the effect of extracellular BCH on the efflux of AIB from MDA-MB-231 and MCF-7 cells. In this set of experiments, AIB efflux was first measured from cells incubated in amino acid-free buffers followed by buffers supplemented with varying concentrations of BCH (Fig. 4a and b). It is apparent that BCH *trans*-stimulated AIB efflux from both cell lines in a dose-dependent fashion. Thus, BCH at a concentration of 10, 50, 75, 100 and 2000 μM, respectively, increased AIB efflux (basal-to-peak) from MDA-MB-231 cells by 70.3% ($P<0.05$), 204.4% ($P<0.02$), 216.5% ($P<0.01$), 244.6% ($P<0.001$) and 312.2% ($P<0.01$). Note from Fig. 3a that BCH at a concentration of 1 μM did not have any effect on AIB efflux from MDA-MB-231 cells. BCH at a concentration of 50, 100 and 2000 μM increased the fractional release of AIB (basal-to-peak) from MCF-7 cells by 83.4% ($P<0.01$), 98.4% ($P<0.001$) and 230.3% ($P<0.001$), respectively. In contrast, 10 μM BCH did not significantly *trans*-stimulate AIB release from MCF-7 cells.

4.2.2. The effect of cationic amino acids on AIB efflux

The finding that L-lysine inhibited the BCH-sensitive component of AIB uptake by MDA-MB-231 and MCF-7 cells prompted us to investigate the effects of cationic amino acids on AIB release. L-lysine, but not L-arginine, *trans*-accelerated AIB efflux from MDA-MB-231 cells when tested at a concentration of 2 mM (Fig. 5a). The fractional release of AIB was increased (basal-to-peak) from 0.0119 ± 0.0006 to 0.0358 ± 0.0027 (± S.E., $n=10$, $P<0.001$) by the addition of L-lysine. However, the interaction between L-lysine and AIB efflux appears to be one of relatively low affinity as 0.1 mM L-lysine had no effect on AIB efflux from MDA-MB-231 cells (Fig. 5a). L-lysine (2 mM) also *trans*-stimulated AIB release from MCF-7 cells (Fig. 5b). Thus, the fractional efflux of AIB was increased (basal-to-peak) from 0.0138 ± 0.0014 to 0.0349 ± 0.0027 (± S.E., $n=6$, $P<0.001$). In contrast, L-arginine (2 mM) had no effect on AIB efflux from MCF-7 cells.

4.2.3. AIB efflux is selectively trans-stimulated by external amino acids

We also examined the effect of L-alanine, L-glutamine, L-leucine, L-tryptophan, AIB, MeAIB, L-proline and L-glu-

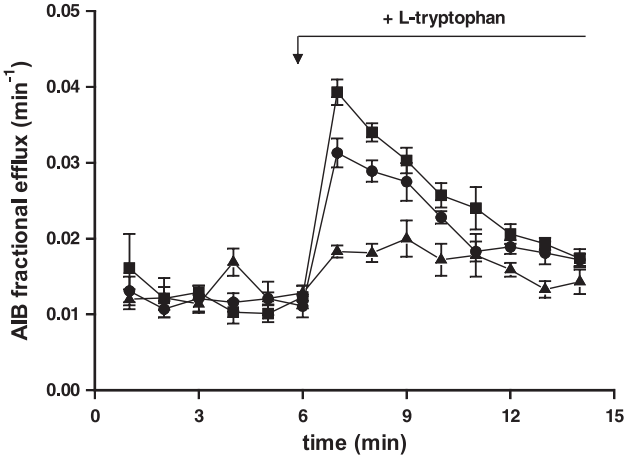

Fig. 7. The effect of external L-tryptophan at 10 μM (▲), 200 μM (●) and 2 mM (■) on AIB efflux from MDA-MB-231 cells. The incubation medium contained (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4 ± L-tryptophan (as indicated). Each point is the mean ± S.E. of between four and six experiments.

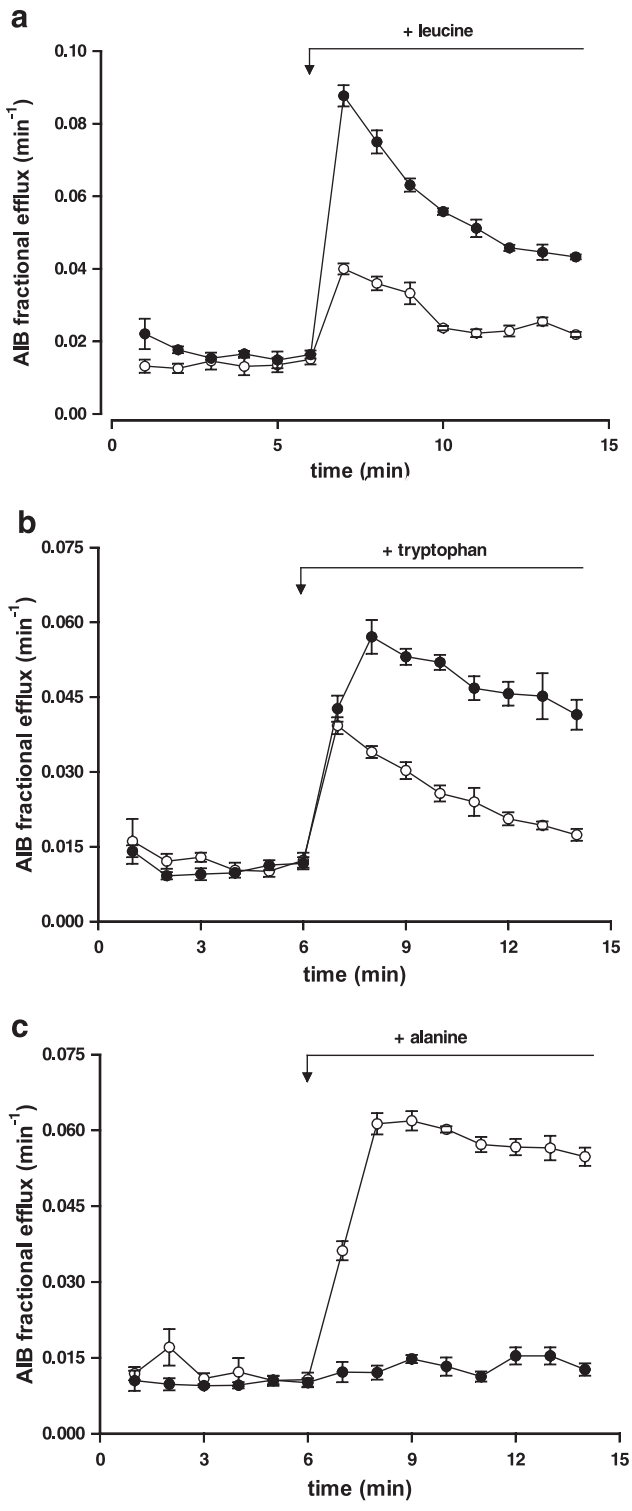

Fig. 8. The effect of external D-leucine (a), D-tryptophan (b) and D-alanine (c) on AIB efflux from MDA-MB-231 cells. Shown for comparison are the effects of their L-isomer counterparts. The data relating to the effects of the D-isomers are represented by the filled circles. The incubation medium contained (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris–MOPS, pH 7.4. When required, the amino acids were used at a concentration of 2 mM. Each point is the mean ± S.E. of four experiments. The data relating to the effect of the L-isomers is the same as that shown in Figs. 6a and 7.

tamine (all tested at 2 mM) on AIB efflux from MDA-MB-231 cells. It is apparent from Fig. 6a that L-alanine ($P<0.001$), L-glutamine ($P<0.01$), L-leucine ($P<0.001$) and AIB ($P<0.02$) all *trans*-stimulated AIB efflux, whereas no effect was found with L-proline, L-glutamate and MeAIB. L-alanine at a concentration of 0.1 mM had no significant effect on AIB release from MDA-MB-231 cells (results not shown). Extracellular L-tryptophan stimulated AIB efflux from MDA-MB-231 cells in a dose-dependent manner (Fig. 7). Thus, L-tryptophan at a concentration of 10, 200 and 2000 μM, respectively stimulated AIB release (basal-to-peak) from MDA-MB-231 cells by 63.5% ($P<0.05$), 181.9% ($P<0.001$) and 219.5% ($P<0.001$). The effect of external amino acids (2 mM) on AIB efflux from MCF-7 cells was also examined (Fig. 6b). L-alanine ($P<0.001$), L-glutamine ($P<0.001$), L-leucine ($P<0.001$) and AIB ($P<0.01$) *trans*-stimulated AIB efflux from MCF-7 cells. On the other hand L-pro, L-glu and MeAIB had no effect.

4.2.4. The effect of D-isomers on AIB efflux

Fig. 8 illustrates the effect of amino acid D-isomers on AIB efflux from MDA-MB-231 cells. For comparison, the effects of their L-isomer counterparts are also shown. It is evident that D-tryptophan ($P<0.002$) and D-leucine ($P<0.001$) effectively *trans*-accelerated AIB efflux. Indeed, at a concentration of 2 mM, it appears that D-tryptophan and D-leucine are more effective than their L-isomer counterparts. In contrast, D-alanine did not *trans*-stimulate AIB efflux from MDA-MB-231 cells. D-leucine (2 mM) also stimulated AIB release from MCF-7 cells: the effect was greater than that found with L-leucine (results not shown).

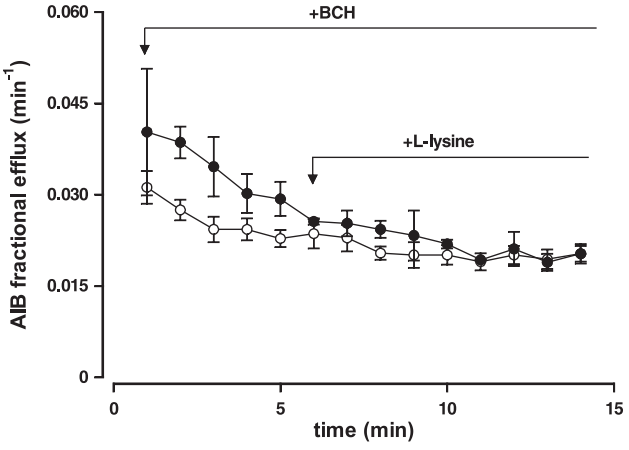

Fig. 9. The effect of external L-lysine in the presence of external BCH on AIB efflux from MDA-MB-231 (●) and MCF-7 cells (○). Efflux was measured into a medium containing (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 10 Tris–MOPS, pH 7.4 and 2 BCH. External L-lysine was used at a concentration of 2 mM. Each point represents the mean ± S.E. of three and four observations, respectively, when MDA-MB-231 and MCF-7 cells were used.

4.2.5. BCH, L-lysine and D-leucine share a pathway for transport

It is clear that both BCH and L-lysine stimulate AIB efflux from MDA-MB-231 and MCF-7 cells. However, BCH and L-lysine could either stimulate AIB efflux via a common or separate pathways. To distinguish between the two possibilities we examined the effect of L-lysine in the presence of BCH. Thus, we measured AIB efflux from cells incubated first in a medium containing BCH followed by one containing both BCH and L-lysine. The results of these experiments are shown in Fig. 9. It is clear that L-lysine loses the capacity to *trans*-stimulate AIB efflux from MDA-MB-231 and MCF-7 cells in the presence of BCH which is consistent with the notion that both amino acids share a pathway(s) for transport. In addition, it is apparent that D-leucine and BCH share a pathway for transport in MDA-MB-231 cells given that the efficacy of D-leucine to *trans*-stimulate AIB efflux was markedly diminished in the presence of BCH (results not shown).

4.2.6. Molecular identity of system L in MDA-MB-231 and MCF-7 cells

We subjected total RNA isolated from MDA-MB-231 and MCF-7 cells to RT-PCR to test for the presence of LAT1, LAT2 and CD98 (Fig. 10). Human placental tissue was used as a positive control since it has been reported that placental trophoblast expresses all three transcripts [23]. Products for the expected size for LAT1, LAT2 and CD98 were amplified from MCF-7 cells. In contrast, only LAT1 and CD98 mRNA was found in MDA-MB-231 cells.

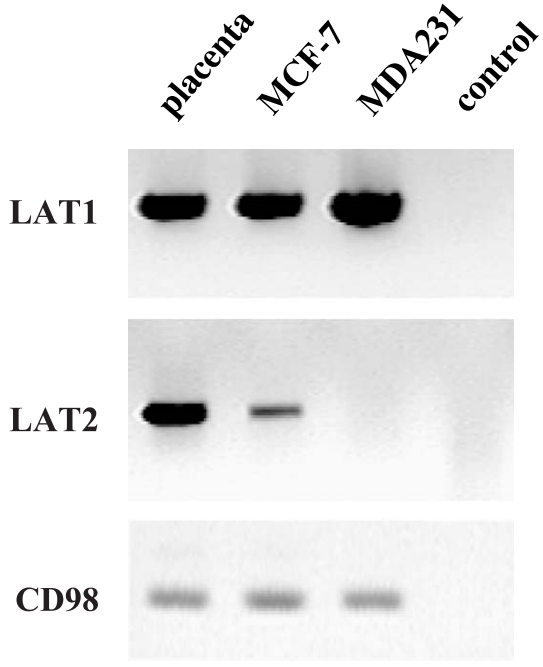

Fig. 10. LAT1, LAT2 and CD98 RT-PCR products from human placenta, MCF-7, MDA-MB-231 cells. The control track represents PCR in the absence of cDNA.

5. Discussion

5.1. Evidence for system L activity in MDA-MB-231 and MCF-7 cells

The present study was undertaken to characterise the functional and molecular properties of system L in cultured human breast cancer cell lines namely, MDA-MB-231 and MCF-7 cells. Given that we wanted to study amino acid efflux as well as influx, we used AIB as a substrate and thus the confounding issue of intracellular metabolism was avoided. The use of AIB is valid given that this non-metabolizable amino acid analogue interacts with system L in a variety of cell types [24–26]. In the present study, system L activity was defined as BCH-sensitive AIB transport measured under Na^+-free conditions. BCH is regarded as a relatively specific substrate of system L. Based on these criteria, the results clearly show that AIB uptake into both cell lines is via system L. Indeed, under our experimental conditions, system L appears to be the major, if not the sole, pathway for AIB uptake into MDA-MB-231 and MCF-7 cells.

The results show that BCH, when added to the incubation medium, stimulated the efflux of AIB from MDA-MB-231 and MCF-7 cells. This suggests that system L in these mammary cancer cell lines acts as an exchange mechanism. This is in agreement with the findings that both LAT1 and LAT2, when co-expressed with CD98, act as amino acid exchangers and not uniporters [10]. Moreover, the stimulation of AIB efflux from the mammary cell lines by extracellular BCH suggests that BCH is actually transported by system L and is not simply acting as a *cis*-inhibitor of amino acid uptake. Other amino acids including L-glutamine, L-leucine, L-alanine, and AIB acted to *trans*-accelerate AIB efflux from MDA-MB-231 and MCF-7 cells. Thus, it appears that system L has a relatively wide substrate specificity in these cell lines. The effect of extracellular amino acids was, however, specific since MeAIB, L-glutamate and L-proline had no effect on AIB release.

Although it is apparent that system L in MDA-MB-231 and MCF-7 cells can operate as an exchanger, the question which arises is: does system L have to act as an obligatory amino acid exchanger? In this connection, it is interesting to note that there was a significant efflux of AIB from both cell types even in the absence of extracellular amino acids. If this flux is mediated via system L, then it would appear that the transporter is able to operate as a uniporter. However, at present, we cannot rule out the possibility that AIB efflux under zero-*trans* conditions is via a pathway(s) other than the L system.

5.2. System L in mammary cancer cell lines interacts with L-lysine

One interesting feature about the present study is the finding that L-lysine interacts with AIB transport in MDA-

MB-231 and MCF-7 cells. Thus, AIB influx and efflux was respectively inhibited and stimulated by extracellular L-lysine, suggesting that system L in MDA-MB-231 and MCF-7 cells accepts L-lysine as a substrate. At first sight, the interaction between AIB transport and L-lysine could be taken as evidence for transport via system y^+L. However, this can be ruled out on the basis that system y^+L is not inhibited by BCH even at a concentration of 5 mM [27]. We are confident that L-lysine is transported by system L in the mammary cell lines because of the following reasons. First, AIB uptake is almost exclusively via a BCH-sensitive pathway. Therefore, the finding that L-lysine inhibited AIB uptake suggests that BCH and L-lysine are acting at a common locus. Secondly, the effect of L-lysine on AIB efflux cannot be attributed to a stimulation of AIB efflux via a membrane potential-sensitive pathway because L-arginine had no effect. Thirdly, extracellular L-lysine did not stimulate AIB efflux from MDA-MB-231 cells in the presence of BCH, suggesting that both amino acids are acting at a common site. However, we must stress that the interaction between L-lysine and system L appears to be one of relatively low affinity. In this connection, it is striking that the onset of *trans*-stimulation of AIB efflux by L-lysine is much slower than that elicited by BCH: this may relate to the finding that L-lysine interacts with system L with low affinity. However, the sequential interaction between two transport systems cannot at this stage be completely ruled out.

5.3. Molecular identity of system L in MDA-MB-231 and MCF-7 cells

As mentioned above, LAT1 and LAT2 when co-expressed with CD98 exhibit system L-like activity. It is apparent that MCF-7 cells express both LAT1 and LAT2, whereas MDA-MB-231 cells only express LAT1. How does the functional data compare with what is known about the properties of LAT1 and LAT2? Let us consider the data relating to the MDA-MB-231 cells first because this appears to be the simplest case as they only express one molecular form of system L. LAT1 when co-expressed with CD98 in *Xenopus* oocytes stimulates the transport of aromatic and large neutral amino acids such as L-leucine, L-phenylalanine, L-tryptophan and BCH in a Na^+-independent fashion. In addition, LAT1 transports D-isomers of certain amino acids [4]. Thus, LAT1 could account for the functional properties of AIB transport in MDA-MB-231 cells. However, the finding that AIB influx and efflux was respectively inhibited and stimulated by extracellular L-alanine appears at first sight to be somewhat puzzling. One of the major distinguishing features between LAT1 and LAT2 is that only the latter accepts L-alanine as a substrate at physiological concentrations (e.g. see Ref. [8]). In this connection, it must be borne in mind that the interaction between L-alanine and AIB efflux from MDA-MB-231 cells was one of relatively low affinity. Another difference between the functional properties of system L in MDA-MB-231 cells and those of LAT1 is that the former does not appear to transport D-tryptophan. Kanai et al. [4] have shown that LAT1 displays marked stereospecificity to tryptophan, whereas the present results indicate that both L and D-tryptophan are transported via system L in MDA-MB-231 cells. The functional properties of AIB transport in MDA-MB-231 cells and those of LAT1 appear to differ with respect to the interaction with L-lysine. LAT1 does not transport L-lysine when expressed in *Xenopus* oocytes, whereas our results show that the BCH-sensitive pathway does interact with L-lysine, albeit with low affinity, in a manner which suggests that L-lysine can be considered a substrate.

The relationship between the functional and molecular data in MCF-7 cells is harder to interpret given that both LAT1 and LAT2 are expressed. This is a situation similar to that found in the lactating rat mammary gland [28]. However, the finding that D-leucine was effective at stimulating AIB efflux suggests that LAT1 plays a role in AIB transport. It is notable that the K_m of AIB uptake into MCF-7 cells was higher than the value calculated for MDA-MB-231 cells. The higher K_m may reflect transport of AIB via LAT2 given that LAT2 has a lower affinity than LAT1 for some neutral amino acids (e.g. see Ref. [8]).

Taken together, the results are consistent with the notion that system L may play an important role in providing mammary cancer cells with amino acids. The results also suggest that LAT1 is the molecular correlate of system L in MDA-MB-231 cells. On the other hand, system L activity in MCF-7 cells may be attributed to both LAT1 and LAT2. The observation that MCF-7 cells express LAT1 and LAT2, whereas MDA-MB-231 cells express only LAT1 may be of some clinical significance. If the expression of the amino acid transporters differs between estrogen receptor-positive and -negative tumours in a similar fashion, then the pattern of LAT1/LAT2 expression could be used as an additional diagnostic tool.

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