

Inhibition of system L (LAT1/CD98hc) reduces the growth of cultured human breast cancer cells

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Abstract. It has been suggested that system L (LAT1/CD98hc) is up-regulated in cancer cells, including breast tumour cells, and is therefore a promising molecular target to inhibit or limit tumour cell growth. In view of this, we have examined the effect of BCH and other inhibitors of system L on the growth of MCF-7, ZR-75-1 and MDA-MB-231 cells. Treating cells with BCH markedly inhibited the metabolism of WST-1 in a dose-dependent fashion. Similarly, melphalan and D-leucine inhibited the growth of cultured breast cancer cells whereas MeAIB, an inhibitor of system A, was without effect. The effects of BCH and melphalan on cell growth were non-additive suggesting that both compounds were acting at a single locus. The results indicate that system L is required to maintain MCF-7, ZR-75-1 and MDA-MB-231 cell growth and support the notion that LAT1/CD98hc may be a suitable target to inhibit breast cancer progression.

Introduction

Cells need an adequate supply of amino acids to survive. Thus, amino acids are required for protein and glutathione synthesis, energy metabolism and the regulation of cell volume. A large array of membrane transporters, each operating with distinct substrate specificity, ion dependence and kinetics, are the main route for amino-nitrogen uptake by cells. One such mechanism, system L, has received considerable attention because it mediates the movement of several essential amino acids and therefore has the potential to play an important role in cell growth and metabolism. System L is a Na⁺-independent neutral amino acid transporter which accepts a broad range of substrates and is inhibited by the non-metabolizable amino acid BCH (2-amino-2-norbornane-carboxylic acid (1,2). System L, however, is not a single transporter: four molecular correlates (LAT1-4) have so far been identified

(3-9). Two of them are heterodimers which consist of a catalytic light chain (LAT1 or LAT2) joined to a heavy chain (CD98hc, also known as 4F2hc) by a disulfide bond.

There is growing evidence to suggest that system L is a good therapeutic target to limit the proliferation of tumour cells (10-13). The first link between LAT1 and cancer arose from the study of Wolf *et al* (14) who showed that LAT1 is expressed in colorectal cancer but not in normal colon. There is now evidence to show that the expression of LAT1 may be involved in the progression of liver tumour lesions (15,16), esophageal adenocarcinoma (17,18), astrocytic tumours (12,13), carcinoma of the upper urinary tract (19), lung adenocarcinoma (20) and oral cancer (21). There is also an abundance of evidence showing that LAT1/CD98 is highly expressed in a large variety of cancer cell lines (22). RNA interference of LAT1 inhibits the growth of cultured human oral cancer cells (11). In this connection Baniasadi *et al* (23) have recently reported that BCH is able to affect the expression of genes in T24 human bladder carcinoma cells which are associated with cell survival. Furthermore, the transport of amino acid related compounds via system L (LAT1 CD98hc) is showing great potential as a means of detecting tumours (24,25).

There is a growing body of evidence suggesting that system L also plays an important role in the progression of breast cancer: it has been reported that expression of LAT1 and CD98 in human breast cancers correlates with poor prognosis (26,27). In this connection, we have shown that cultured human breast cancer cells express system L at the functional and molecular level (28-30). Thus, MDA-MB-231 cells express LAT1/CD98hc whereas MCF-7 cells express both LAT1/CD98hc and LAT2/CD98hc. However, the kinetic properties of amino acid transport suggest that the contribution of LAT2 to system L activity in MCF-7 cells is very small compared with the contribution of LAT1 (29). It appears that system L may be the most important pathway for the uptake of certain essential neutral amino acids such as L-leucine, L-phenylalanine and L-tryptophan by cultured human breast cancer cells and is therefore in a position to play a pivotal role in controlling cell growth (28,29).

The purpose of this study was to examine the importance of system L (LAT1) in relation to the growth of breast cancer cell lines. Thus, we examined the effect of various inhibitors of system L (e.g. BCH and melphalan) on the metabolism of WST-1 by MCF-7, ZR-75-1 and MDA-MB-231 cells.

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Materials and methods

Materials. MCF-7 and MDA-MB-231 breast adenocarcinoma cells were purchased from the European Animal Cell Culture Collection. ZR-75-1 cells were bought from LGC Protochem, WST-1 was obtained from Roche, and Dulbecco's modified Eagle medium (DMEM) was purchased from Invitrogen. All other chemicals including BCH, D-leucine and melphalan were purchased from Sigma.

General cell culture conditions. MCF-7, ZR-75-1 and MDA-MB-231 cells were routinely cultured in DMEM supplemented with L-glutamine (2 mM), heat-inactivated fetal bovine serum (10%), penicillin (50 IU/ml) and streptomycin (50 µg/ml). The cells were incubated in a gas phase of air with 5% CO₂ at 37°C. Cells were seeded in 75 cm² flasks at a density of 2.25x10⁶ cells per flask and were cultured in 12 ml of medium for 3-4 days.

Cell culture conditions for cell growth studies. Cells which had been routinely cultured as described above were harvested and seeded in either 6 (for direct cell counts) or 96- (for WST-1 assay) well plates at a density of 3x10⁵ and 1.5x10⁵ cells per well, respectively. The cells were then incubated in phenol red-free DMEM for 24 h (2 ml per 6 well and 100 µl per 96 well). Following this, the medium was changed and the cells were incubated for a period of either 24 or 48 h in phenol red-free DMEM ± test substances (i.e. BCH, D-leucine, MeAIB, melphalan, sucrose or urea) before cell growth was measured using the WST-1 assay or by direct cell counting.

Cell growth assay using WST-1. Cell growth was assayed using a tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol]-1, 3-benzene disulfonate). WST-1 (10 µl) was added to each well which contained 100 µl of culture medium. The cleavage of WST-1 to formazan by metabolically active cells was quantified after 3 h of incubation by measuring the optical density at 450/690 nm using a Tecan Sunrise absorbance reader. Culture medium containing WST-1 (1/10 v/v) was used as a background control. Absorbance in the wells containing the treated cells was expressed as a percentage of the absorbance of control cells containing untreated cells.

Direct cell counting using the trypan blue exclusion method. MCF-7 cells were seeded in 6-well plates at a density of 3x10⁵ cells per well and cultured in phenol red-free DMEM as described above. The cells in each well were harvested in 0.5 ml of a trypsin/EDTA solution and counted by the blue staining method using a hemocytometer.

Data presentation and statistics. Values shown are means ± SE. For convenience, the data shown in the tables and figures are given as percentages. However, non-transformed data was used for statistical analysis either by Student's t-test or ANOVA. Student's paired t-test was used to examine the difference between two means whereas ANOVA followed by Tukey's test (using GraphPad Prism 4.0) was used when multiple comparisons were required. Differences were considered significant at P<0.05.

Table I. The effect of BCH on WST-1 metabolism by MCF-7 cells.

[BCH] mM	MCF-7 cell growth (% of control) at t=24 h	MCF-7 cell growth (% of control) at t=48 h
0	100	100
1	98.4±3.7 (3)	93.5±2.5 (4)
10	93.0±3.9 (3)	81.8±1.5 (4) ^a
25	75.3±3.6 (4) ^a	67.8±4.5 (4) ^a
50	55.0±2.1 (4) ^a	45.1±2.0 (4) ^a

The data are expressed as % of control (i.e. WST-1 metabolism in the absence of any BCH). The numbers in parentheses represent the number of experiments with each concentration of BCH. Cells were incubated in the presence of BCH for either 24 or 48 h. WST-1 metabolism was assayed after 3 h of incubation at 450/690 nm. ^aP<0.0001 vs. control.

Table II. The effect of BCH on WST-1 metabolism by MDA-MB-231 cells.

[BCH] mM	MDA-MB-231 cell growth (% of control) at t=24 h	ZR-75-1 cell growth (% of control) at t=24 h
0	100	100
1	103.4±2.8 (3)	91.6±7.7 (5)
10	95.6±2.0 (3)	73.7±5.3 (5) ^a
25	69.9±1.1 (4) ^b	59.6±5.9 (5) ^b
50	47.7±1.8 (4) ^b	47.1±2.7 (5) ^b

The data are expressed as % of control (i.e. WST-1 metabolism in the absence of any BCH). The numbers in parentheses represent the number of experiments with each concentration of BCH. Cells were incubated in the presence of BCH for 24 h. WST-1 metabolism was assayed after 3 h of incubation at 450/690 nm. ^aP<0.01; ^bP<0.001 vs. control.

Results

The effect BCH, D-leucine and melphalan on breast cancer cell growth. If system L plays a crucial role in supporting the growth of cultured breast cancer cells it can be predicted that BCH should inhibit the metabolism of WST-1. To test this prediction we examined the effect of BCH (1-50 mM) on the growth of MCF-7, ZR-75-1 and MDA-MB-231 cells. It is apparent from the results shown in Table I that MCF-7 cell growth was inhibited by BCH in a dose-dependent manner when tested over a period of 24 and 48 h. BCH significantly inhibited MCF-7 cell growth over a period of 24 h when present at a concentration of 25 and 50 mM. When tested over 48 h, BCH significantly reduced MCF-7 cell proliferation when used at a concentration of 10, 25 and 50 mM. Similarly, the results in Table II suggest that the growth of MDA-MB-231 and ZR-75-1 cells over a period of 24 h was also inhibited by BCH in a dose-dependent manner. WST-1 metabolism by ZR-75-1 cells was significantly reduced by 10, 25 and 50 mM BCH whereas MDA-MB-231 cell growth was significantly inhibited by 25 and 50 mM BCH.

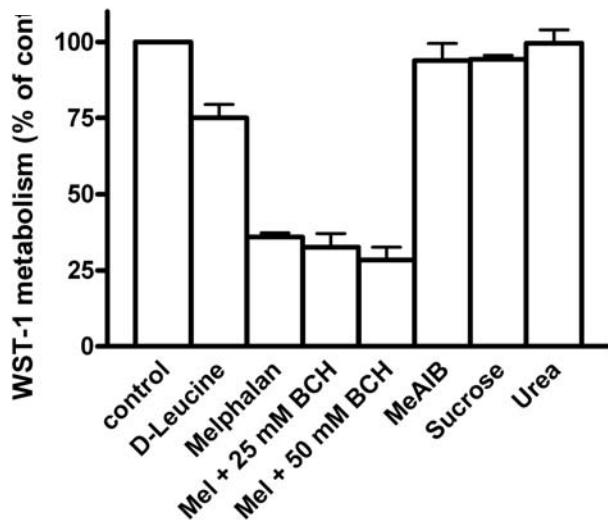


Figure 1. The effect of D-leucine, melphalan, melphalan plus BCH (Mel + BCH), MeAIB, sucrose and urea on MCF-7 cell growth measured using the WST-1 assay. Data are expressed as % of control. Cells were incubated in the presence/absence of the various compounds for 24 h. WST-1 metabolism was assayed after 3 h of incubation at 450/690 nm. D-leucine, sucrose and urea were used at a concentration of 50 mM. BCH was used at a concentration of 25 or 50 mM and melphalan was used at a concentration of 100 μ M. Data shown are the means \pm SE of 3-5 experiments.

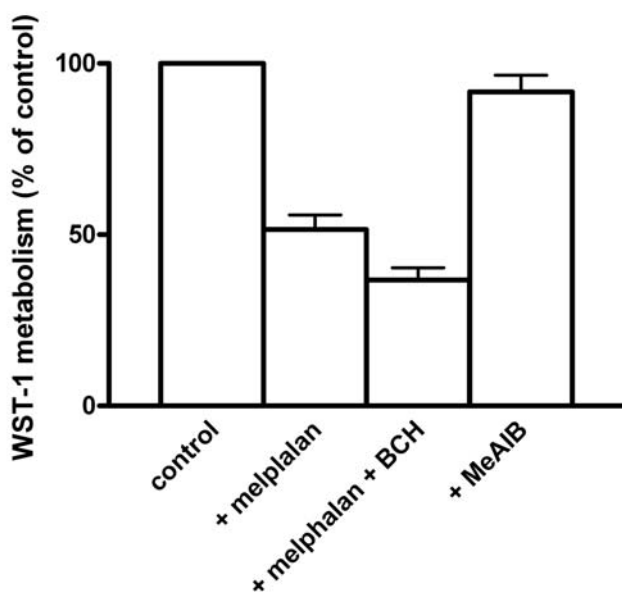


Figure 2. The effect of melphalan (100 μ M), melphalan + BCH (100 μ M and 50 mM, respectively) and MeAIB (50 mM) on ZR-75-1 cell growth measured using the WST-1 assay. Data are expressed as % of control. Cells were incubated in the presence of the compounds for 24 h. WST-1 metabolism was assayed after 3 h of incubation at OD 450/690 nm. Data shown are the means \pm SE of 4 experiments.

We tested the effect of D-leucine (50 mM) and melphalan (100 μ M) on the metabolism of WST-1 by MCF-7 as both compounds have been shown to inhibit L-leucine uptake by cultured breast cancer cells (29). D-leucine and melphalan respectively inhibited MCF-7 cell growth by $24.9 \pm 4.3\%$

($P < 0.05$) and $64.1 \pm 1.4\%$ ($P < 0.001$) (Fig. 1). It is notable from the results shown in Fig. 1 that the effects of BCH and melphalan were not additive. Thus, a combination of melphalan and BCH (at either 25 or 50 mM) did not significantly reduce WST-1 metabolism by MCF-7 cells compared to that found with melphalan alone. In contrast, MeAIB (methylaminoisobutyric acid), a paradigm substrate of system A, had no effect on the metabolism of WST-1 by MCF-7 cells (Fig. 1). Furthermore, MCF-7 cell growth was unaffected by either 50 mM sucrose or 50 mM urea (Fig. 1). Similarly, sucrose had no effect on the metabolism of WST-1 by MDA-MB-231 cells (results not shown).

We also examined the effect of melphalan and MeAIB on the metabolism of WST-1 by ZR-75-1 cells (Fig. 2). Melphalan (100 μ M) reduced cell growth by $48.4 \pm 4.3\%$ ($P < 0.001$). BCH (50 mM) in the presence of melphalan (100 μ M) reduced WST-1 metabolism by $63.2 \pm 3.5\%$ ($P < 0.001$); therefore, the inhibition of ZR-75-1 cell growth by melphalan and BCH was not additive. In contrast, MeAIB (50 mM) did not significantly reduce ZR-75-1 cell growth (Fig. 2).

The effect of BCH on MCF-7 cell growth was also quantified by direct cell counting using a hemocytometer. Culturing cells in the presence of BCH at a concentration of 50 mM for 24 h reduced the cell number by $41.5 \pm 5.7\%$ ($P < 0.01$).

Discussion

System L may be the major, if not sole pathway for the uptake of certain essential amino acids such as L-leucine, L-phenylalanine and L-tryptophan into cultured breast cancer cells, suggesting that it may play an important role in regulating breast cancer cell growth (28,29). Consistent with this notion, we now show that BCH, albeit at relatively high concentrations, markedly reduced the metabolism of WST-1 by MCF-7, ZR-75-1 and MDA-MB-231 cells. We also found that BCH reduced the MCF-7 cell number to a similar extent suggesting that measuring WST-1 metabolism is an acceptable method to quantify the effect of system L inhibitors on breast cancer cell growth. On the basis of our previous results we predicted that BCH would inhibit transport via LAT1/CD98hc.

It may, at first sight, appear odd that a relatively large concentration of BCH was required to inhibit MCF-7, ZR-75-1 and MDA-MB-231 cells growth given that we have shown that BCH is a high affinity inhibitor of radio-labelled amino acid transport into human breast cancer cells (28,29). However, the culture medium used in our experiments contained relatively high levels of amino acids which are known to be substrates of system L. Consequently, relatively large concentrations of BCH would be required to reduce the net uptake of amino acids thus inhibiting cell growth. In accordance with our findings, Nawashiro *et al* (12) found that a concentration of >25 mM BCH was required to inhibit the growth of C6 glioma cells. The conclusion that BCH exerts its affect on the growth of cultured breast cancer cells by restricting the uptake of amino acids (especially essential amino acids) which are substrates of system L is supported by our findings relating to the effect of D-leucine on MCF-7 cell growth. D-leucine, like BCH, is a non-metabolizable

substrate of LAT1/CD98hc (29), that is expected to limit the availability of essential amino acids.

Consistent with previous results, we found that melphalan inhibited the growth of MCF-7 cells (31). The effects of melphalan and BCH on MCF-7 cell growth were not additive suggesting that they are acting at the same locus (i.e. LAT1/CD98hc). Similarly, melphalan and BCH appear to inhibit ZR-75-1 cell growth at a common locus. There is evidence to suggest that melphalan enters its target cells, including MCF-7 cells, via system L (32). However, we and others have suggested that the rate of melphalan transport via LAT1/CD98 is slow compared to naturally occurring amino acids and thus should be viewed as an effective inhibitor of system L. (29,33). In this connection, it is notable that MCF-7 cells selected for their resistance to melphalan grow at a slower rate than their wild-type counterpart (32). This could be a consequence of reduced LAT1/CD98h activity. Indeed, down-regulation of CD98 expression in melphalan resistant myeloma cells has been reported (34). On the basis of the present results, it is tempting to speculate that melphalan exerts its action on cancer cells by restricting the movement of essential amino acids across the plasma membrane.

The effect of BCH, D-leucine and melphalan appears to be relatively specific given that MeAIB, an inhibitor of system A, a transporter which is present in MCF-7 cells (35), did not significantly reduce WST-1 metabolism by MCF-7 cells. Moreover, the effect of BCH and D-leucine on cell growth cannot simply be attributed to an increase in the osmolarity of the medium because sucrose (50 mM) had no effect on WST-1 metabolism. In addition, the finding that urea, a compound that readily permeates plasma membranes had no effect on cell growth suggests that the effect of BCH and D-leucine was not due to an increase in the intracellular osmolarity.

Ring *et al* (26) have shown that the expression of LAT1 in breast cancer is a potentially important prognostic immunohistochemical marker for estrogen receptor positive breast cancer. Furthermore, the expression of CD98hc in primary breast cancer cells correlates with poor patient prognosis (27). Therefore, the effect of BCH, D-leucine and melphalan found in the present study adds weight to the argument that system L (LAT1/CD98hc) is an attractive target to limit or reduce the growth of breast cancer cells.

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