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Impact of system L amino acid transporter 1 (LAT1) on proliferation of human ovarian cancer cells: A possible target for combination therapy with anti-proliferative aminopeptidase inhibitors

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ABSTRACT

Amino acids activate nutrient signaling via the mammalian target of rapamycin (mTOR), we therefore evaluated the relationship between amino acid transporter gene expression and proliferation in human ovarian cancer cell lines. Expression of three cancer-associated amino acid transporter genes, LAT1, ASCT2 and SN2, was measured by qRT-PCR and Western blot. The effects of silencing the *LAT1* gene and its inhibitor BCH on cell growth were evaluated by means of cell proliferation and colony formation assays. The system L amino acid transporter LAT1 was up-regulated in human ovarian cancer SKOV3, IGROV1, A2780, and OVCAR3 cells, compared to normal ovarian epithelial IOSE397 cells, whereas ASCT2 and SN2 were not. BCH reduced phosphorylation of p70S6K, a down-stream effector of mTOR, in SKOV3 and IGROV1 cells, and decreased their proliferation by 30% and 28%, respectively. Although proliferation of SKOV3 (S1) or IGROV1 (I10) cells was unaffected by LAT1-knockdown, plating efficiency in colony formation assays was significantly reduced in SKOV3(S1) and IGROV1(I10) cells to 21% and 52% of the respective plasmid transfected control cells, SKOV3(SC) and IGROV(IC), suggesting that LAT1 affects anchorage-independent cell proliferation. Finally, BCH caused 10.5- and 4.3-fold decrease in the IC_{50} value of bestatin, an anti-proliferative aminopeptidase inhibitor, in IGROV1 and A2780 cells, respectively, suggesting that the combined therapy is synergistic. Our findings indicate that LAT1 expression is increased in human ovarian cancer cell lines; LAT1 may be a target for combination therapy with anti-proliferative aminopeptidase inhibitors to combat ovarian cancer.

1. Introduction

Tumor cells exhibit increased demand for a variety of nutrients due to their rapid growth. Among these nutrients, amino acids are particularly important not only as building blocks for protein synthesis, but also as activators of the mTOR (mammalian target of rapamycin) in a nutrient signaling pathway, which in turn regulates cell-survival pathways that are hyperactive in many cancers [1]. Among the 20 amino acids, leucine, an essential amino acid, has been shown to be the most effective to stimulate protein synthesis through nutrient signaling pathways independent of insulin [2]. Therefore, intracellular amino acid availability may be a key factor for cell-survival in tumor tissues because the activity of mTOR is often up-regulated in human cancers [3].

Mammalian cells have a broad range of mechanisms for transmembrane transport of amino acids [4]. Tumor cells have an increased demand for essential amino acids as well as glutamine [5,6]. It is known that some amino acid transporters are up-regulated in cancer cells, presumably to support the high levels of protein synthesis necessary for rapid cell proliferation [7,8]. LAT1 (*SLC7A5*) is the prototype of the system L type of amino acid transporters; it heterodimerizes with the 4F2 heavy chain (termed

Abbreviations: LAT1, System L amino acid transporter; BCH, 2-aminobicyclo[2.2.1]-heptane-2-carboxylic acid; mTOR, mammalian target of rapamycin; p70S6K, p70 S6 kinase; SRB, sulforhodamine B; PI3K, phosphatidylinositol-3-kinase; ASCT2, system ASC amino acid transporter 2; SN2, system N amino acid transporter 2; HPRT, hypoxanthine phosphoribosyltransferase 1; RT-PCR, reverse transcription polymerase chain reaction; XTT, (2,3)-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide.

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as CD98, or *SLC3A2*), and the complex imports large neutral amino acids including essential amino acids such as leucine in exchange for intracellular neutral amino acids in a Na^+-independent manner [9,10]. Among the amino acid transporters, increased expression in various types of human cancer cells has been well documented for LAT1 [10]. Since immunostaining of LAT1 directly correlates with malignancy of glioblastoma, LAT1 is thought to be a potential molecular target for cancer chemotherapy [11]. ASCT2 (*SLC1A5*) is an amino acid transporter responsible for transport of neutral amino acids including glutamine, leucine and isoleucine [12]. Expression of ASCT2 is essential for cell growth and viability of human hepatoma cells in which ASCT2 is associated with translational machinery through mTOR for cell growth [13,14]. In addition, SN2 (*SLC38A5*), a Na^+-dependent transporter for neutral amino acids (e.g. glutamine and asparagine) [15,16], has been reported to be associated with human cancerous tissues [8]. Therefore, it is of clinical importance to understand whether the functional expression of these amino acid transporters has any bearing on clinicopathological variables since they may provide us with novel drug targets for cancer therapy or diagnostic marker.

The AKT/mTOR signaling is often enhanced in human epithelial ovarian cancer, and thought to be attractive target for the therapy [17]. Since there is no standard maintenance therapy for ovarian cancer, we became interested in mTOR signaling mediated via nutrient pathways. However, little information is available concerning the relationship of amino acid transporter expression and cell growth in human ovarian cancer. In the present study, we investigated expression of the *LAT1*, *ASCT2*, and *SN2* genes in various human ovarian cancer cell lines compared to their normal counterparts, human immortalized ovarian surface epithelial IOSE397 cells and ovarian tissues. These studies have shown that LAT1 is up-regulated in ovarian cancer cell lines and that the transporter has a significant impact on their colony-forming ability. Furthermore, we examined the efficacy of 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH), a competitive inhibitor of LAT1 to enhance the anti-proliferative effects of bestatin, an aminopeptidase inhibitor that blocks amino acid recycling from breakdown of proteasome-generated peptides by intracellular amino peptidases. Our studies find that BCH potentiates the anti-tumor activity of bestatin, suggesting a combination therapy with bestatin and a LAT1 inhibitor may be effective for the treatment of ovarian cancer.

2. Materials and methods

2.1. Materials

Human ovarian cancer cell lines SKOV3, IGROV1, OVCAR3, A2780 and human acute myeloid leukemia cell line HL-60 were obtained from American Type Culture Collection (ATCC, Manassas, VA). MV4-11 cell line was obtained from Dr. Angelika Burger, Wayne University (Detroit, MI). Human immortalized ovary surface epithelial cell line IOSE397, a model for normal human ovarian epithelium, was provided generously by Dr. Nelly Auersperg (University of British Columbia, Vancouver, Canada) [18]. RPMI1640 medium, minimum essential medium (MEM) and heat-inactivated fetal bovine serum (FBS) were purchased from Mediatech, Inc. (Manassas, VA), Invitrogen (Carlsbad, CA) and Atlantic Biologicals (Miami, FL), respectively. BCH, bestatin, puromycin, non-essential amino acids, sulforhodamine B and all other reagents for the cell culture were purchased from Sigma–Aldrich (St. Louis, MO). The Cell Proliferation Kit II using XTT was obtained from Roche Applied Science (Indianapolis, IN). All double-strand oligodeoxynucleotides used in this study were supplied through Biopolymer/Genomics Shared Service at University of Maryland at Baltimore. For quantitative RT-PCR (qRT-PCR), TRIzol and PCR reagents were purchased from Invitrogen and Applied Biosystems (Carlsbad, CA), respectively. Retroviral vector MSCV/LTRmirR30-PIG, and a retrovirus packaging cell line, LinX, were obtained from Open Biosystems (Huntsville, AL).

2.2. Cells

Human ovarian cancer and IOSE397 cells were maintained in RPMI1640 medium supplemented with 10% heat-inactivated FBS. IOSE397 cells were cultured as described previously [19]. For amino acid depletion, amino acid deprived MEM was prepared based on the formulation provided for minimum essential medium from Invitrogen (Carlsbad, CA).

2.3. LAT1-knockdown

To develop ovarian cancer cell lines with knockdown of *LAT1*, double-strand DNA containing a hairpin sequence for the targeted site (5′- cggtacgaatctcatccctcaa-3′) of LAT1 mRNA was inserted into the microRNA-adapted retroviral vector MSCV/LTRmirR30-PIG. The plasmid vector was transfected into a retrovirus packaging cell line, LinX, and viral particles produced into the supernatant were collected by filtration. SKOV3 and IGROV1 cells were infected, and the next day selection was started with 2 μg/mL of puromycin. Finally, a single clone from SKOV3 and two clones from IGROV1 were established, and designated S1, and I6 and I10, respectively. As control, we established SKOV3 (SC) and IGROV1 (IC) cell lines transduced with the plasmid DNA containing an oligonucleotide not related to any genes.

2.4. Western blot

In general, cells of interest were washed with ice-cold PBS and homogenized in RIPA buffer (50 mM Tris–HCl, pH 7.5; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA; 1 mM sodium orthovanadate; 1 mM NaF; 10 mM sodium pyrophosphate; 1 μg/mL leupeptin; 1 μg/mL aprotinin; and 2 mM Pefabloc SC [Roche Applied Science, Indianapolis, IN]) by sonication. For the stimulation assay of mTOR activity, cells were deprived of FBS overnight and further deprived of amino acids for 2 h, then stimulated with amino acids for 30 min in the presence or the absence of BCH, a competitive inhibitor of LAT1. Lysates from these cells were used for Western blot. Debris from lysates was removed by centrifugation. The protein concentration of the resultant supernatant was determined by the method of Bradford [20]. A 25 μg aliquot of total cell lysate was subjected to SDS–polyacrylamide gel electrophoresis and then electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The blots were then probed with anti-LAT1 polyclonal antibody (Cosmo Bio, Carlsbad, CA) at 1–200 dilution, anti-p70S6K rabbit monoclonal antibody, or anti-phospho-p70 S6K (Thr389) mouse monoclonal antibody (Cell Signaling Technology, Danvers, MA), followed by appropriate secondary antibodies conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured cells of interest using TRIzol reagent. Snap-frozen samples of normal human ovary tissue were obtained from the University of Maryland Greenebaum Cancer Center Tissue Bank in accordance with protocols approved by the University of Maryland School of Medicine Institutional Review Board. Generally, DNase-treated RNA was subjected to reverse transcription reaction for 60 min at 37 °C followed by up to 35 PCR cycles of denaturation at 95 °C for 1 s, annealing at 60 °C for

15 s, and elongation at 72 °C for 15 s. The gene-specific primers used to detect LAT1, ASCT2, SN2 and CD98 were as follows: LAT1, sense, 5′-cacagaaagcctgagcttga-3′ and antisense, 5′-cacctgcatgagcttctga-3′; ASCT2, sense, 5′-tggtctcctggatcatgtgg-3′ and antisense, 5′-tttgcgggtgaagaggaagt-3′; SN2, sense, 5′-tgaaacacttgggctacc-3′ and antisense, 5′-gggcactgtgtaggaca-3′; CD98, sense, 5′-ttggctgagtggcaaaatatca-3′ and antisense, 5′-gattcgagtaggctcaggatctg-3′. The sequences of primers for HPRT were reported previously [21]. The absolute expression levels of LAT1 mRNA were determined by qRT-PCR using LightCycler® (Roche Applied Science, Indianapolis, IN) from a standard curve created by the linear calibration of the crossing points obtained with known concentrations of authentic LAT1 cDNA template. The expression level was normalized to that of HPRT.

2.6. Leucine uptake by ovarian cancer cells

Ovarian cancer cells (1.0×10^5 cells/well) were plated onto a 24-well plate two days before uptake experiment. In general, uptake experiment was initiated by adding 0.25 mL of transport buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES, adjusted to pH 7.4) containing 16 nM [^{3}H]leucine (Moravek Biochemicals and Radiochemicals, Brea, CA), and then cells were incubated for 1 min at 37 °C. At the end of the uptake reaction, cells were washed off the substrates with ice-cold transport buffer twice, and then solubilized in 0.2 mL of 1% (v/v) Triton X-100 (Sigma–Aldrich). The radioactivity in the resultant cell lysate was measured using a liquid scintillation counter (Aloka, Tokyo, Japan). Part of the lysate was used for determination of total protein amount with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Uptake rate was evaluated by obtaining cell-to-medium (CM) ratio (μL/mg protein) by dividing the intracellular accumulation of [^{3}H]leucine by its concentration in the transport medium.

2.7. Cell proliferation assay

The effects of BCH and bestatin on proliferation of adherent ovarian cancer cells were evaluated by measuring total protein staining with sulforhodamine B (SRB assay) as described previously [22]. In general, 500 cells were plated with 0.2 mL of culture medium into a 96-well tissue culture plate. The cells were cultured for 6 days at 37 °C in an atmosphere of 5% CO_2, fixed with 10% trichloroacetic acid and stained with 0.057% (w/v) SRB. SRB was dissolved in 0.1 mL of 10 mM Tris/HCl (pH 10), and then the absorbance of each well at 570 nm (and at 630 nm as a reference) was measured using a microplate reader. The Cell Proliferation Kit II was used for floating cells (e.g. HL60 and MV4-11 cells) according to the manufacturer's instructions.

2.8. Colony formation assay

To test for colony formation in soft agar, 2000 cells were suspended in 0.4 mL of RPMI1640 containing 0.35% agar (DNA grade, Sigma–Aldrich) with or without 20 mM BCH. Cells were then seeded onto a 0.5% agar base (0.4 mL) in 24-well tissue culture plates. After 2–3 weeks, colonies were stained with iodonitrotetrazolium chloride (1 mg/mL in PBS, Sigma–Aldrich) and the number of colonies was counted using a tumor colony counter (Microbiology Systems International, Frederick, MD).

2.9. Statistical analysis

Student's *t*-test was used to assess the significance of difference between *in vitro* assay results, with $p < 0.05$ as a criterion of significance. To estimate IC_{50} values of bestatin in ovarian cancer cells, cell proliferation assay data were fitted to the Hill equation with a slope of −1.0.

3. Results

3.1. Expression of amino acid transporters in human ovarian cancer cells

LAT1 mRNA expression as quantified by qRT-PCR in five human ovarian cell lines was 22-, 29-, 24-, 11-, and 10-fold greater in the cancer cell lines SKOV3, IGROV1, OVCAR3, and A2780 and the immortalized normal human ovarian epithelial cell line IOSE397 than that in human normal ovary, respectively (Fig. 1A left). The levels of mRNA for ASCT2 and SN2, two other amino acid transporters known to be associated with cancerous tissues [8], were examined by means of conventional RT-PCR. No significant increase in mRNA expression of ASCT2 was observed among ovarian cancer cells, while SN2 mRNA was detected only in the normal ovarian cell line IOSE397 and, to a much lesser extent, in normal human ovarian tissue (Fig. 1A right). To verify these findings, ASCT2 and SN2 mRNA expression were quantified using real-time qRT-PCR. ASCT2 mRNA expression in SKOV3 and IGROV1 cells was 76% and 94% of that in IOSE397 cells, respectively, whereas SN2 expression decreased to 7% and 4% (as mean value of 2 individual PCR results).

CD98 mRNA expression was also examined because heterodimerization of LAT1 with CD98 is obligatory for plasma membrane targeting of the transporter. The levels of CD98 mRNA were slightly higher in SKOV3, IGROV1 and OVCAR3 cells (Fig. 1A right) than in IOSE397 cells. Therefore, we further examined LAT1 protein expression in both forms, the LAT1 monomer (35 kDa) and the LAT1/CD98 heterodimer (125 kDa), in all five cell lines by means of Western blot (Fig. 1B). The expression of both forms was increased markedly in the four ovarian cancer cell lines compared to that in IOSE397 cells.

Furthermore, in order to evaluate LAT1 function in these cell lines, [^{3}H]leucine uptake by ovarian cancer cells was measured in the absence (control) and the presence of BCH, a competitive inhibitor of LAT1 that blocks LAT1-mediated entry of amino acids into cells (Fig. 1C). In the absence of BCH, [^{3}H]leucine uptake by SKOV3, IGROV1, and A2780 cells was 3.96-, 3.32-, and 2.21-fold greater than that of IOSE397 cells, respectively (Fig. 1C, control). [^{3}H]Leucine uptake was decreased to 10.4%, 11.3%, and 14.1% of control uptake in SKOV3, IGROV1, and A2780 cells, respectively, in the presence of 1 mM BCH, whereas the uptake was almost completely inhibited with 10 mM BCH in all cells lines. These results suggest that LAT1 is functionally up-regulated in human ovarian cancer cell lines, and that BCH is effective in inhibiting leucine uptake in these lines.

3.2. Inhibition of mTOR signaling pathway in ovarian cancer cell lines by BCH, a competitive inhibitor of LAT1

To determine whether LAT1-mediated amino acid-transport in human ovarian cancer cell lines plays a regulatory role in relation to mTOR activity, we measured phosphorylation of p70S6K (T389), a down-stream effector of mTOR, in SKOV3 and IGROV1 cells. As shown in Fig. 2, exposure of the cells to amino acids induced the phosphorylation of p70S6K in both lines. This effect was attenuated to a significant extent in the presence of 10 mM BCH. The levels of total p70S6K remained unchanged. Statistical analysis of the band intensities obtained from three individual densitometric scans of the Western blots showed a significant reduction in phosphorylation of p70S6K in SKOV3 cells (41 ± 6% vs. control). These results suggest a significant role of LAT1-mediated amino acid-transport in the stimulation of mTOR signaling pathway.

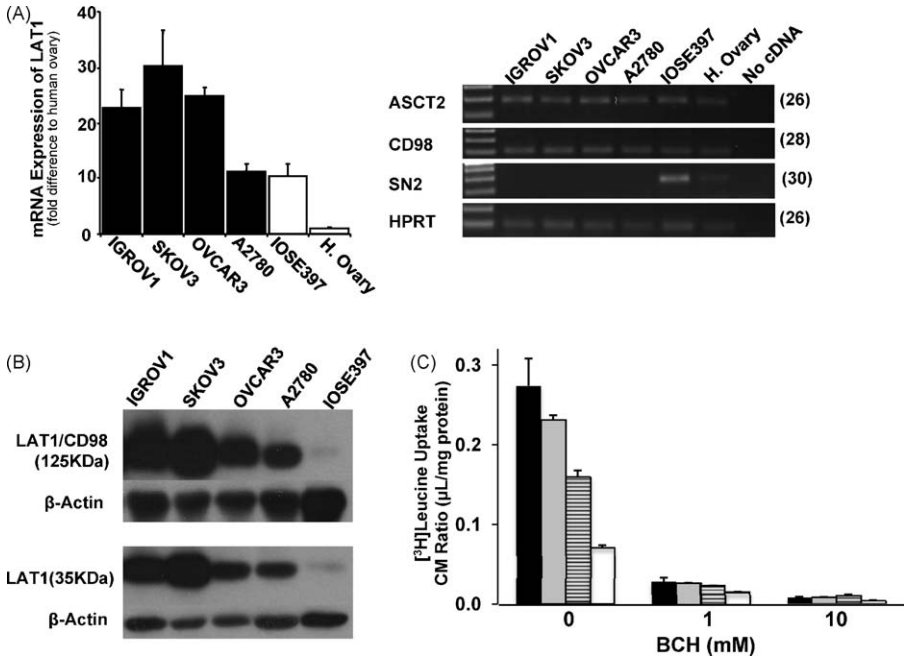

Fig. 1. Expression levels of amino acid transporters in human ovarian cancer cell lines. (A, left) mRNA expression levels of LAT1 were determined by qRT-PCR. Each bar represents mean ± S.E. of at least three individual results. (A, right) mRNA expression of ASCT2, SN2, CD98 and HPRT was determined by RT-PCR. PCR products were visualized on 2% agarose gel. Number shown in parenthesis indicates cycle number. The figure represents three individual RT-PCR results. (B) Western blot analysis of LAT1/CD98 heterodimer (125 kDa) and LAT1 monomer (35 kDa). The heterodimer and monomer were detected in cell lysates denatured in the absence or the presence of DTT. The blot represents at least three individual Western blots. (C)[^{3}H]Leucine uptake by SKOV3 (black), IGROV1 (grey), A2780 (hatched) and IOSE397 (white) cells was measured at pH 7.4 and 37 °C for 1 min. Experiment was repeated twice and each bar represents the mean ± S.E. (n = 4).

3.3. Impact of LAT1 inhibitors or LAT1-knockdown on cell proliferation

Since mTOR signaling was inhibited significantly by BCH, we examined the effect of BCH on proliferation of SKOV3, IGROV1 and IOSE397 cells. Cell proliferation was monitored for 6 days in the presence or the absence of BCH. BCH at a concentration of 1 mM had no effect on cell growth in all three lines examined (Fig. 3A). However, when the concentration was increased to 10 mM, BCH slightly but significantly reduced cell growth in SKOV3 (~30%) and IGROV1 (~28%). Under similar conditions, BCH had no effect on the growth of IOSE397 cells (Fig. 3A). In order to evaluate the involvement of LAT1 in the growth of ovarian cancer cell lines unequivocally, the expression of LAT1 was silenced with a gene-specific shRNA in both SKOV3 and IGROV1 cells. Western blots showed that LAT1 protein expression decreased significantly in the cloned LAT1-knockdown sublines SKOV3(S1), IGROV1(I6 and I10) compared to that in the control cell lines SKOV3(SC) and IGROV1(IC) (Fig. 3B). However, when cell proliferation was compared between LAT1-knockdown cells and control lines for 5 days, no significant difference was observed (Fig. 3C), similar to the minimal effects of BCH on proliferation (Fig. 3A). Furthermore, phosphorylation of p70S6K was not significantly altered in S1 and I10 cells (data not shown). These results suggest that LAT1-mediated amino acid-transport has no significant effect on the proliferation of these human ovarian cancer cell lines growing as adherent monolayers in liquid culture.

We then examined the effects of BCH on the ability of SKOV3 and IGROV1 cells to form colonies using the soft agar colony formation assay. In contrast to the cell proliferation assay in liquid culture, the clonogenic assay revealed that BCH significantly reduced plating efficiency in control cells (72% reduction in SC cells and 84% reduction in IC cells) (Fig. 3D). The plating efficiency of the LAT1-knockdown cells S1, I6 and I10 decreased to 21%, 38% and 52% of the corresponding control, respectively. In the presence of 10 mM BCH, the colony-forming ability of these cells with LAT1-knockdown was significantly reduced, indicating that the amino acid supply via LAT1 may be crucial for anchorage-independent growth of ovarian cancer cells.

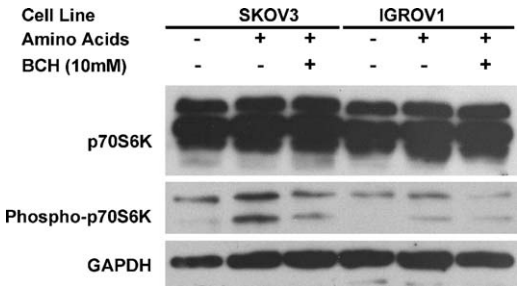

Fig. 2. Influence of LAT1 inhibition on phosphorylation of p70S6K in SKOV3 and IGROV1 cells. Cells were treated with or without amino acids in the absence or the presence of BCH. Cell lysates were prepared as described in Section 2. Blots were probed by anti-p70S6K rabbit monoclonal antibody (top), anti-phospho-p70 S6K (Thr389) mouse monoclonal antibody (middle), or anti-GAPDH rabbit monoclonal antibody (bottom). Anti-p70S6K antibody detects p85S6K (upper band), and anti-phospho-p70 S6K (Thr389) antibody detects phospho-p85S6K (Thr412). The blot represents at least three individual results.

3.4. Treatment of human ovarian cancer or leukemia cells with BCH combined with bestatin, an aminopeptidase inhibitor

Intracellular pools of amino acids are maintained by cellular amino acid uptake mediated by transporters such as LAT1, and by amino acid recycling, predominantly mediated by breakdown of proteasome-generated peptides to free amino acids by cellular aminopeptidases. Since LAT1 inhibition or knockdown had only a limited effect on anchorage-dependent cell proliferation in human ovarian cancer cell lines, we tested whether combining BCH with an aminopeptidase inhibitor would more efficiently deprive cancer cells of amino acids. The anti-proliferative effect of the aminopeptidase inhibitor bestatin was measured in the presence or the absence

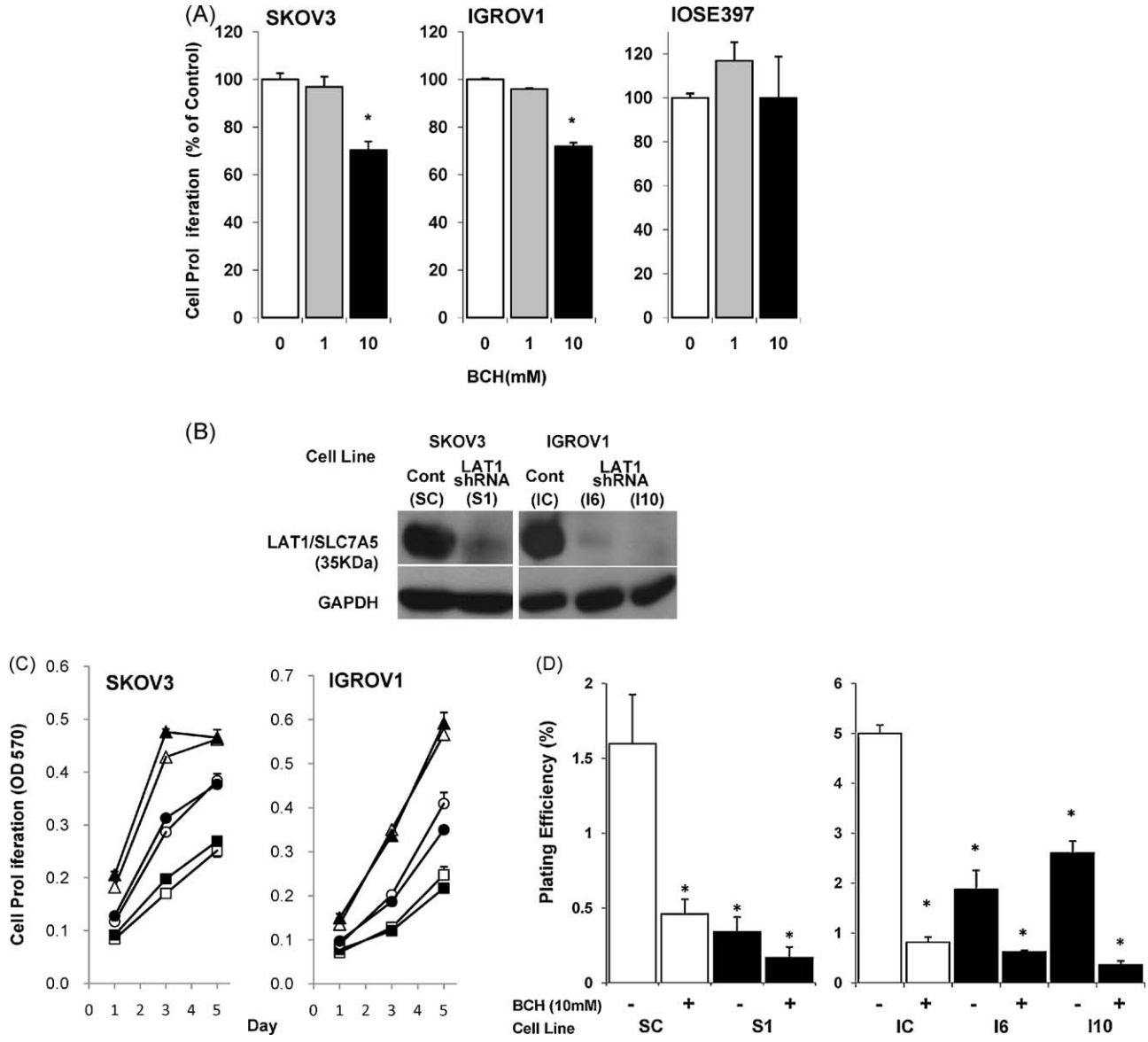

Fig. 3. Impact of LAT1-mediated amino acid-transport on cell proliferation and colony formation. (A) The effect of BCH on cell proliferation was investigated in SKOV3, IGROV1, and IOSE397 cells, which were seeded at 500, 500 and 5000 cells/well, respectively, in a 96-well tissue culture plate on Day 0. After 6 days proliferation was determined by performing SRB assay. Each bar (mean ± S.E.) represents proliferation relative to the control (in the absence of BCH). Experiments were repeated at least twice in quadruplicate in the absence or the presence of 1 mM or 10 mM BCH. $^*p < 0.05$ vs. control by Student's *t*-test. (B) Western blot analysis of LAT1 monomer (35 kDa) in control and LAT1-knockdown S1, and I6 and I10 cells. The blot represents at least three individual results. (C) Proliferation of S1 (left) and I10 (right) cells was monitored for up to 5 days by SRB assay with cells seeded at 500 cells/well (square), 1000 cells/well (circle), and 2000 cells/well (triangle). Open symbols show results for the controls (SC or IC cells), and closed symbols for S1 or I10 cells. Each point (mean ± S.E.) represents proliferation (OD 570). Experiments were repeated at least three times in quadruplicate. (D) Colony formation assay was carried out as described in Section 2. Open bars show the plating efficiency results for controls (SC or IC cells). Other results are for S1 (black), or I6 or I10 cells (black). Data are given as mean ± S.E. Experiments were repeated at least twice in triplicate. $^*p < 0.05$ vs. the control in the absence of BCH by Student's *t*-test.

of 1 or 10 mM BCH in the human ovarian cancer cell lines (Fig. 4A). At both concentrations of BCH applied, cell growth was more markedly decreased in the presence of both bestatin and BCH than in the presence of either one alone in all three human ovarian cancer cell lines (SKOV3, IGROV1, and A2780), but not in the normal human ovarian epithelial IOSE397cells. Bestatin has been reported to be a potent inhibitor of leukemia cell growth [23]. To compare to the effects we observed in ovarian cancer cell lines with those in leukemia cell lines, we examined the effects of BCH on proliferation of two human leukemia cell lines (HL60 and MV4-11). As shown in Fig. 4B left, LAT1 expression was detected in both cell lines in heterodimer as well as monomer forms, albeit at lower expression levels than in the ovarian cancer line. A synergistic effect of BCH in combination with bestatin was evident in both HL60 and MV4-11 cells because cell proliferation was completely suppressed when the two compounds are used in combination (Fig. 4B right). The IC_{50} values of bestatin measured in IGROV1 and A2780 cell lines decreased to 14.3 ± 2.4 μM (10.5-fold) and 43.5 ± 4.1 μM (4.3-fold) with 10 mM BCH, compared to the corresponding values without BCH (150 ± 16.1 μM and 189 ± 10.31 μM, respectively) (Fig. 4C). Such an effect was not observed in IOSE397 cells (13.7 ± 2.8 μM in the presence of BCH vs. 6.4 ± 2.2 μM in the absence of BCH). To determine whether LAT1-knockdown alters sensitivity to bestatin, cell proliferation of LAT1-knockdown in IGROV1 cells, I10, was measured in the presence of bestatin. Cell proliferation of I10 cells significantly decreased about two fold in the presence of 300 μM bestatin, which inhibited cell proliferation of IGROV1 cells to about 20% in the presence of bestatin, compared to that of IC cells (Fig. 4D). These data suggest that LAT1 has potential as a drug target in the treatment of ovarian cancer for combination therapy with an aminopeptidase inhibitor.

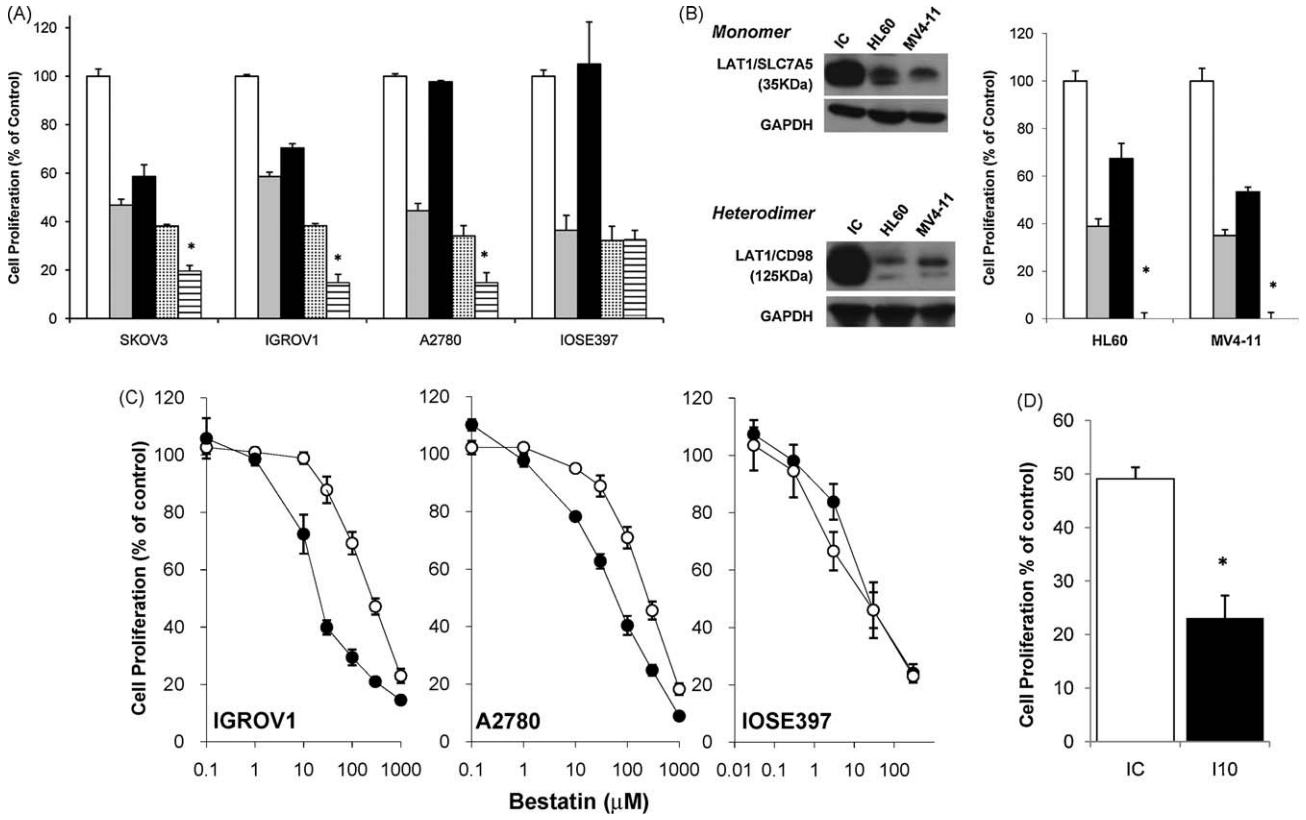

Fig. 4. BCH enhances anti-proliferative effect of amino peptidase inhibitor bestatin. (A) Cell proliferation was studied in control medium (white), or in the presence of bestatin (1 mM, grey), 10 mM (black) BCH, or both (1 mM BCH [dotted] or 10 mM BCH [hatched] with bestatin) in three ovarian cancer cell lines (SKOV3, IGROV1 and A2780) and IOSE397 by monitoring cell proliferation for 6 days. Cells were seeded at a density of 500 cells/well for cancer cell lines, and 5000 cells/well for IOSE397 cells on Day 0. Each bar (mean ± S.E.) represents proliferation relative to the control (in the absence of both agents). Experiments were repeated at least twice in triplicate. $^*p < 0.05$ vs. control by Student's *t*-test. (B) Western blot analysis of LAT1/CD98 heterodimer (125 kDa) and LAT1 monomer (35 kDa) in human leukemia HL60 and MV4-11 cells. As control, cell lysates from IC cells were used for the blot. The blot represents at least three individual results. Cell proliferation was studied in control medium (white), or in the presence of bestatin (300 μM, grey), BCH (10 mM, black) or both (hatched) in two leukemia cell lines, HL-60 and MV4-11 cells, for 6 days. Cells were seeded at a density of 500 cells/well on Day 0. Each bar (mean ± S.E.) represents proliferation relative to the control (in the absence of both agents). Experiments were repeated at least twice in triplicate. $^*p < 0.05$ vs. the control by Student's *t*-test. (C) Anti-proliferative activity of bestatin at different concentrations in the absence (open circle) or the presence (closed circles) of 10 mM BCH for IGROV1 (left), A2780 (middle), and IOSE 397 (right) cells. Each point represents mean ± S.E. Experiments were repeated at least twice in quadruplicate. (D) Effect of LAT1-knockdown on cell proliferation of IGROV1 cells in the presence of bestatin (300 μM) was investigated in IC and I10 cells. Both cells were plated at 500 cells/well, respectively, in a 96-well tissue culture plate on Day 0. After 6 days proliferation was determined by performing SRB assay. Each bar (mean ± S.E., $n = 8$) represents proliferation relative to the control (in the absence of bestatin). $^*p < 0.05$ vs. control by Student's *t*-test.

4. Discussion

4.1. Amino acid transporters in cancer cells

LAT1 has been reported to be useful as a prognostic marker in malignant tumor tissues such as astrocytoma and glioma [11]. Since LAT1 recognizes essential neutral amino acids with bulky side chains including leucine and isoleucine, blocking LAT1-mediated amino acid-transport could have a significant impact on cell proliferation. The present study using cell lines provides a rationale for combination therapy with a LAT1 inhibitor and an aminopeptidase inhibitor for the treatment of ovarian cancers, and also possibly leukemias in which LAT1 is up-regulated.

In the last decade, a number of studies have shown that expression of several amino acid transporters is up-regulated in tumor tissues, suggesting an essential role for amino acid supply in supporting the high rate of proliferation in cancer cells. Increased expression of the system L transporter LAT1 is well documented in various types of human cancer cells, including leukemia [10], breast cancer [24], colon cancer [25], glioma [11], oral cancer [26] and esophageal carcinoma [27]. Our data showing the upregulation of LAT1 in human ovarian cancer cell lines (Fig. 1) are in good agreement with the recent observation in human epidermal ovarian cancer cells, in which LAT1 was significantly up-regulated in surgically resected ovarian tumor tissue specimens obtained from patients, compared to benign ovarian tumors, suggesting that increased LAT1 expression contributes to cell proliferation and migration of ovarian cancer [28]. However, mRNA expression of LAT1 in IOSE397 cells was relatively higher than human ovarian tissues. Since the cell line has been immortalized by transfection of normal OSE cells with SV40 large T antigen [18], it may not necessarily reflect transcriptional regulation for the *LAT1* gene which occurs in human ovarian epithelium.

In contrast to LAT1, increased expression of ASCT2, another leucine transporter, was not observed in ovarian cancer cell lines. The expression of another amino acid transporter (SN2) was examined because upregulation of SN2 was found in cancer tissues based on a search of the human expressed sequence tag database as a part of the Cancer Genome Anatomy Project using the "cDNA Virtual Northern" tool [8]. Interestingly, our data show a significant decrease or loss in SN2 mRNA expression in at least four different human ovarian cancer cell lines compared to normal ovarian tissue based on the results from RT-PCR (Fig. 1A right). This could be due to a compensation effect for LAT1 upregulation. This observation is new and additional work is needed to determine if the expression of SN2 could be a diagnostic or prognostic marker for ovarian cancer.

4.2. Role of LAT1 in cancer cell proliferation

Addition of the LAT1 inhibitor BCH (10 mM) into culture medium reduced cell proliferation in both SKOV3 and IGROV1 cells by only about 30%. This is in agreement with a previous report showing that *in vitro* proliferation of C6 rat glioma cells was inhibited by approximately 40% in the presence of 10 mM BCH [11]. LAT1 recognizes and transports leucine with a Km value of 20 μM [29], and the reported IC_{50} value of BCH to inhibit leucine uptake by human cancer cell lines expressing LAT1 is ~180 μM in the breast cancer cell line MCF-7 [24] and ~150 μM in oral keratinocytes [26]. Based on these values, BCH at a concentration of 10 mM, the concentration employed in the present study, is expected to cause a significant blockade of LAT1-mediated amino acid-transport. Indeed 10 mM BCH almost completely inhibited leucine uptake by ovarian cancer cells (Fig. 1C). Unexpectedly, LAT1-knockdown did not cause a significant retardation in anchorage-dependent cell growth of both SKOV3 and IGROV1 cells in liquid medium (Fig. 3C). It is possible that the loss of LAT1 could be compensated either by upregulation of other amino acid transporters or by amino acid recycling catalyzed by aminopeptidase. Although BCH is a relatively specific inhibitor to LAT1, it also inhibits other system L transporters including LAT2 (*SLC7A8*) to a lesser extent [6], and β-alanine carrier system amino acid transporter $ATB^{0,+}$, which accepts leucine [30]. Since our data show little effect of LAT1-knockdown on cell proliferation, other transporters or biological factors may be involved in the moderate effect of BCH on cell proliferation that may not be mediated by LAT1. Hence, as a single entity, LAT1-mediated amino acid-transport may have a limited impact on cell proliferation in tumor tissues.

4.3. LAT1 and anchorage-independent cell growth

The present study provides the first evidence that blockade of LAT1 is efficient in inhibiting anchorage-independent growth of human ovarian cancer cell lines, suggesting an important role for LAT1 in cell-survival under the non-adhesive conditions as involved in processes such as cancer cell migration and metastasis. Consistent with a recent study by Yamauchi et al. in squamous cell carcinoma of the head and neck [31], we found that BCH also inhibits the phosphorylation of p70S6K in SKOV3 and IGROV1 ovarian cancer cells (Fig. 2). Modulation of mTOR activity by alterations in intracellular amino acid availability may affect anchorage-independent growth of ovarian cancer cells. Among various oncogenic signaling pathways, phosphatidylinositol-3-kinase (PI3K)/AKT is the most important for anchorage-independent growth [32]. The PI3K/AKT pathway utilizes multiple downstream effectors including p70S6K. A previous report showed that the colony-forming ability of rat intestinal epithelial cells, transformed by an oncogenic insulin receptor, was inhibited by the mTOR inhibitor rapamycin as well as by the PI3K inhibitor LY294002 [33], implying a regulatory role for mTOR in anchorage-independent growth. Since we found that the phosphorylation of p70S6K was completely abolished by 10 μM LY29004 in SKOV3 cells (data not shown), LAT1 may at least play a role in uncontrolled growth and metastasis of malignantly transformed cells *in vivo*. Additional studies are warranted to clarify how PI3K signaling via mTOR promotes anchorage-independent growth of human ovarian cancer cell lines.

4.4. Possibility of combination therapy with anti-proliferative aminopeptidase inhibitors

Bestatin is an aminopeptidase inhibitor, first isolated from culture filtrate of *Streptomyces olivoreticuli* [34], and it exhibits anti-proliferative effects on animal [35,36] and human [37] tumors via potentiation of host immune responses. In the present study, bestatin exerted anti-proliferative activity in human ovarian cancer cell lines (IGROV1 and AG2780) with IC_{50} values of 150–190 μM. These values are similar to the IC_{50} values for human leukemia cell lines HL60 and U937 [38,39], indicating that bestatin functions as a potent anti-proliferative agent also in ovarian cancer cells. Recent studies have shown that the mechanism of bestatin action as a anti-proliferative agent in human leukemia cell lines HL60 and U937 involves deprivation of cancer cells of amino acids due to blockade of protein recycling [38,39].

Amino acid transporters also supply amino acids to cancer cells, and increased LAT1 expression was found in various types of human ovarian cancer cells (Fig. 1). Since our data suggest that LAT1-knock down has little impact on cell proliferation in liquid medium (Fig. 3C), we wanted to determine the relative contribution of BCH-sensitive system L transporters including LAT1, in the situation where the intracellular amino acid availability decreases. BCH, at even 1 mM, significantly suppressed cell proliferation of the all three cancer cells cultured in the presence of bestatin, suggesting that cell-survival becomes more dependent upon amino acid uptake via system L amino acid transporters since 1 mM BCH did not affect cell proliferation in the absence of bestatin. This notion was demonstrated by the potentiating effect of BCH (10 mM), which gives a maximum blockade of amino acid-transport, on the anti-proliferative effect of bestatin in ovarian cancer cells, but not in IOSE397 cells. BCH enhanced sensitivity of IGROV1 and A2780 cells to bestatin-induced inhibition of cell proliferation, reducing the IC_{50} value to 14.3 and 43.5 μM, respectively (Fig. 4C). Finally, cell proliferation of the LAT1-knockdown IGROV1 clone I10 significantly decreased in the presence of bestatin (Fig. 4D). This suggests that at least lowering LAT1 expression causes the sensitivity to bestatin. Hence, LAT1 may be a potential target for the combination therapy with anti-proliferative amino peptidase inhibitors when cancer cells are deprived of amino acids for therapeutic purposes.

The maximum plasma concentration of bestatin in healthy human volunteers after oral administration at a single dose of 30 mg was reported about 7 μM [40]. Therefore, bestatin concentrations around the IC_{50} values obtained could be achievable *in vivo*; in contrast, the BCH concentrations used (up to 10 mM) were quite high. These experimental findings raise the possibility that co-administration of inhibitor of system L amino acid transporters including LAT1 with aminopeptidase inhibitors may be effective for the prevention of cell proliferative activity. Since our data suggest a striking effect of BCH on anchorage-independent cell growth, such inhibitor would also be expected to be efficient in preventing metastatic activity of microinvasive cancer cells in ovarian tumors, particularly if more efficient inhibitors for LAT1 are developed.

Recently, a novel, orally bioavailable agent, CHR-2797, has been reported to display much more potent, tumor cell-selective, anti-proliferative properties to human leukemia cells, than bestatin [38]. It would be worthwhile to examine the ability of a LAT1 inhibitor to potentiate the anti-proliferative effect of CHR-2797 on leukemia as well as ovarian cancer cells.

5. Conclusion

In conclusion, our present study shows that LAT1 is up-regulated in several human ovarian cancer cell lines, but not in normal ovarian epithelial cell line or in normal ovarian tissue. Although blocking LAT1-mediated amino acid-transport had only limited effect on anchorage-dependent cell proliferation of ovarian cancer cells, it caused a striking inhibition of anchorage-independent growth. Furthermore, our studies showed that the combina-

tion of a LAT1 inhibitor with an aminopeptidase inhibitor may be a new and promising strategy targeting cellular amino acid pools as a way to combat ovarian cancer and leukemia.

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References

[1] Christie GR, Hajduch E, Hundal HS, Proud CG, Taylor PM. Intracellular sensing of amino acids in Xenopus laevis oocytes stimulates p70 S6 kinase in a target of rapamycin-dependent manner. J Biol Chem 2002;277:9952–7.
[2] Greiwe JS, Kwon G, McDaniel ML, Semenkovich CF. Leucine and insulin activate p70 S6 kinase through different pathways in human skeletal muscle. Am J Physiol Endocrinol Metab 2001;281:E466–71.
[3] Bjornsti M-A, Houghton PJ. The TOR pathway: a target for cancer therapy. Nat Rev Cancer 2004;4:335–48.
[4] Palcin M, Eetevez R, Bertran J, Zorzano A. Molecular biology of mammalian plasma membrane amino acid transporters. Physiol Rev 1998;78:969–1054.
[5] Rivera S, Azcon-Bieto J, Lopez-Soriano J, Miralpeix M, Argiles J. Amino acid metabolism in tumour-bearing mice. Biochem J 1988;249:443–9.
[6] Christensen HN. Role of amino acid transport and countertransport in nutrition and metabolism. Physiol Rev 1990;70:43–77.
[7] Nakanishi T. Drug transporters as targets for cancer chemotherapy. Cancer Genom Proteom 2007;4:241–54.
[8] Fuchs BC, Bode BP. Amino acid transporters ASCT2 and LAT1 in cancer: partners in crime? Semin Cancer Biol Tumor Metab 2005;15:254–66.
[9] Kanai Y, Segawa H, Miyamoto K-i, Uchino H, Takeda E, Endou H. Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 Antigen (CD98). J Biol Chem 1998;273: 23629–32.
[10] Yanagida O, Kanai Y, Chairoungdua A, Kim DK, Segawa H, Nii T, et al. Human L-type amino acid transporter 1 (LAT1): characterization of function and expression in tumor cell lines. Biochim Biophys Acta 2001;1514:291–302.
[11] Nawashiro H, Otani N, Shinomiya N, Fukui S, Ooigawa H, Shima K, et al. L-type amino acid transporter 1 as a potential molecular target in human astrocytic tumors. Int J Cancer 2006;119:484–92.
[12] Kekuda R, Prasad PD, Fei Y-J, Torres-Zamorano V, Sinha S, Yang-Feng TL, et al. Cloning of the sodium-dependent, broad-scope, neutral amino acid transporter Bo from a human placental choriocarcinoma cell line. J Biol Chem 1996;271: 18657–61.
[13] Fuchs BC, Perez JC, Suetterlin JE, Chaudhry SB, Bode BP. Inducible antisense RNA targeting amino acid transporter ATB0/ASCT2 elicits apoptosis in human hepatoma cells. Am J Physiol Gastrointest Liver Physiol 2004;286:G467–78.
[14] Fuchs BC, Finger RE, Onan MC, Bode BP. ASCT2 silencing regulates mammalian target-of-rapamycin growth and survival signaling in human hepatoma cells. Am J Physiol Cell Physiol 2007;293:C55–63.
[15] Nakanishi T, Sugawara M, Huang W, Martindale RG, Leibach FH, Ganapathy ME, et al. Structure, function, and tissue expression pattern of human SN2, a subtype of the amino acid transport system N. Biochem Biophys Res Commun 2001;281:1343–8.
[16] Nakanishi T, Kekuda R, Fei YJ, Hatanaka T, Sugawara M, Martindale RG, et al. Cloning and functional characterization of a new subtype of the amino acid transport system N. Am J Physiol Cell Physiol 2001;281:C1757–68.
[17] Trinh X, van Dam P, Dirix L, Vermeulen P, Tjalma W. The rationale for mTOR inhibition in epithelial ovarian cancer. Expert Opin Investig Drugs 2009;18: 1885–91.
[18] Choi K-C, Kang SK, Tai C-J, Auersperg N, Leung PCK. The regulation of apoptosis by activin and transforming growth factor-{beta} in early neoplastic and tumorigenic ovarian surface epithelium. J Clin Endocrinol Metab 2001;86:2125–35.
[19] Steller MD, Shaw TJ, Vanderhyden BC, Ethier J-F. Inhibin resistance is associated with aggressive tumorigenicity of ovarian cancer cells. Mol Cancer Res 2005;3:50–61.
[20] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[21] Fischer M, Skowron M, Berthold F. Reliable transcript quantification by real-time reverse transcriptase-polymerase chain reaction in primary neuroblastoma using normalization to averaged expression levels of the control genes HPRT1 and SDHA. J Mol Diagn 2005;7:89–96.
[22] Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc 2006;1:1112–6.
[23] Yanagawa T, Oguro M, Takagi T, Takenaga K. Direct antitumor activity of biological response modifiers (B.R.M.) proven by an in vitro sensitivity test. Gan to Kagaku Ryoho 1984;11:2155–62.
[24] Shennan DB, Thomson J, Gow IF, Travers MT, Barber MC. Leucine transport in human breast cancer cells (MCF-7 and MDA-MB-231): kinetics, regulation by estrogen and molecular identity of the transporter. Biochim Biophys Acta (BBA)—Biomembr 2004;1664:206–16.
[25] Wolf DA, Wang S, Panzica MA, Bassily NH, Thompson NL. 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 products. Cancer Res 1996;56:5012–22.
[26] Yoon JH, Kim YB, Kim MS, Park JC, Kook JK, Jung HM, et al. Expression and functional characterization of the system amino acid transporter in KB human oral epidermoid carcinoma cells. Cancer Lett 2004;205:215–26.
[27] Kobayashi H, Ishii Y, Takayama T. Expression of L-type amino acid transporter 1 (LAT1) in esophageal carcinoma. J Surg Oncol 2005;90:233–8.
[28] Kaji M, Kabir-Salmani M, Anzai M, Jin C, Akimoto Y, Horita A, et al. Properties of L-type amino acid transporter 1 in epidermal ovarian cancer. Int J Gynecol Cancer 2010;20:329–36.
[29] Kanai Y, Endou H. Functional properties of multispecific amino acid transporters and their implications to transporter-mediated toxicity. J Toxicol Sci 2003;28:1–17.
[30] Nakanishi T, Hatanaka T, Huang W, Prasad PD, Leibach FH, Ganapathy ME, et al. Na+- and Cl-coupled active transport of carnitine by the amino acid transporter ATB0, + from mouse colon expressed in HRPE cells and Xenopus oocytes. J Physiol (Lond) 2001;532:297–304.
[31] Yamauchi K, Sakurai H, Kimura T, Wiriyasermkul P, Nagamori S, Kanai Y, et al. System L amino acid transporter inhibitor enhances anti-tumor activity of cisplatin in a head and neck squamous cell carcinoma cell line. Cancer Lett 2009;276:95–101.
[32] Wang L-H. Molecular signaling regulating anchorage-independent growth of cancer cells. Mt Sinai J Med 2004;71:361–7.
[33] Nguyen KT, Wang W-J, Chan JL-K, Wang L-H. Differential requirements of the MAP kinase and PI3 kinase signaling pathways in Src- versus insulin and IGF-1 receptors-induced growth and transformation of rat intestinal epithelial cells. Oncogene 2000;19:5385–97.
[34] Umezawa H, Aoyagi T, Suda H, Hamada M, Takeuchi T. Bestatin, an inhibitor of aminopeptidase B produced by actinomycetes. J Antibiot (Tokyo) 1976;29:97–9.
[35] Ishizuka M, Masuda T, Kanbayashi N, Fukusawa S, Takeuchi T, Aoyagi T, et al. Effect of bestatin on mouse immune system and experimental murine tumors. J Antibiot (Tokyo) 1980;33:642–52.
[36] Nakanishi T, Tamai I, Takaki A, Tsuji A. Cancer cell-targeted drug delivery utilizing oligopeptide transport activity. Int J Cancer 2000;88:274–80.
[37] Ota K, Kurita S, yamada K, Masaoka T, Uzuka Y, Ogawa N. Immunotherapy with bestatin for acute nonlymphocytic leukemia in adults. Cancer Immunol Immunother 1986;23:5–10.
[38] Krige D, Needham LA, Bawden LJ, Flores N, Farmer H, Miles LEC, et al. CHR-2797: An antiproliferative aminopeptidase inhibitor that leads to amino acid deprivation in human leukemic cells. Cancer Res 2008;68:6669–79.
[39] Moore HE, Davenport EL, Smith EM, Muralikrishnan S, Dunlop AS, Walker BA, et al. Aminopeptidase inhibition as a targeted treatment strategy in myeloma. Mol Cancer Ther 2009;8:762–70.
[40] Koyama M, Hashimoto M, Asakawa N, Ishibashi M, Miyazaki H. Simultaneous determination of bestatin and p-hydroxybestatin, a major metabolite, in human serum by gas chromatography mass spectrometry. Utilization of quantitative selected ion monitoring and deuterium labelled internal standard. Biomed Mass Spectrom 1980;7:372–6.