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A new treatment for human malignant melanoma targeting L-type amino acid transporter 1 (LAT1): A pilot study in a canine model

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ABSTRACT

L-type amino acid transporter 1 (LAT1), an isoform of amino acid transport system L, transports branched or aromatic amino acids essential for fundamental cellular activities such as cellular growth, proliferation and maintenance. This amino acid transporter recently has received attention because of its preferential and up-regulated expression in a variety of human tumors in contrast to its limited distribution and low-level expression in normal tissues. In this study, we explored the feasibility of using LAT1 inhibitor as a new therapeutic agent for human malignant melanomas (MM) using canine spontaneous MM as a model for human MM. A comparative study of LAT expression was performed in 48 normal tissues, 25 MM tissues and five cell lines established from MM. The study observed LAT1 mRNA levels from MM tissues and cell lines that were significantly ($P < 0.01$) higher than in normal tissues. Additionally, MM with distant metastasis showed a higher expression than those without distant metastasis. Functional analysis of LAT1 was performed on one of the five cell lines, CMeC-1. [^{3}H]L-Leucine uptake and cellular growth activities in CMeC-1 were inhibited in a dose-dependent manner by selective LAT1 inhibitors (2-amino-2-norbornane-carboxylic acid, BCH and melphalan, LPM). Inhibitory growth activities of various conventional anti-cancer drugs, including carboplatin, cyclophosphamide, dacarbazine, doxorubicin, mitoxantrone, nimustine, vinblastine and vincristine, were significantly ($P < 0.05$) enhanced by combination use with BCH or LPM. These findings suggest that LAT1 could be a new therapeutic target for MM.

Introduction

Amino acid transport system L, which includes basolateral amino acid transporters, transports branched or aromatic amino acids to cells in a sodium-independent manner [1–3]. Its role in providing the amino acids necessary for cellular activities makes this system essential for fundamental cellular activities such as cell growth, proliferation and maintenance [4,5]. L-type amino acid transporter 1 (LAT1), one of the molecular isoforms belonging to system L, is highly expressed in wide array of human tumors as well as many tumor cell lines, in contrast to its limited distribution and low-level expression in normal tissues [5–9]. Conversely, L-type amino acid transporter 2(LAT2), another system L isoform, expresses throughout normal tissues [10], but has limited expression in tumors [6]. Because LAT1 expresses extensively in tumors and functional inhibitors for LAT1 reduce tumor cell growth [11,12], considerable attention has been paid recently to LAT1 as a tumor marker and as a new therapeutic target in human medicine.

Human malignant melanoma (MM) is the most common malignant neoplastic disease of the digits. Prompt detection and surgical treatment at an early stage of the disease is important. However, the majority of patients with MM present with an advanced stage with distant metastasis when diagnosed. Although systemic chemotherapy is a standard of care for MM with metastasis in human medicine, there are no chemotherapy protocols with satisfactory outcomes [13]. Because human MM expresses LAT1 at a high level [1], the discovery of new therapeutic approaches aimed at LAT1 is anticipated.

Canine MM is believed to be an ideal model of human MM for clinical and therapeutic studies based not only on similar biological behaviors but also on the high incidence of spontaneous canine MM [14,15]. Therefore, we investigated the feasibility of LAT1 as a therapeutic target in human MM by examining canine MM tissues and cell lines obtained from spontaneous MM dogs.

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Materials and methods

Dogs and materials

Twenty-five dogs with a diagnosis of MM based on histopathological examination were enrolled in this study. Six healthy dogs kept at Kitasato University were also used to obtain normal tissues including salivary gland, pancreas, small intestine, large intestine, thyroid gland, adrenal gland, kidney, lymph node, stomach, lung, liver, spleen, urinary bladder and heart.

All experimental procedures in this study progressed under approval by ethics committees in Kitasato and Rakuno Gakuen Universities (Ethics committee approval numbers were 10-028 and VH24A13, respectively).

Tissue samples

Twenty-five MM samples were obtained by excisional surgery from 25 canine MM patients. MM was histopathologically diagnosed by a veterinary pathologist. For molecular analysis, tissue samples were immersed in RNAlater solution (Applied Biosystems, Foster City, CA, USA) overnight at 4 °C, and stored at −80 °C after removal from the solution. For protein analysis, tissue samples were stored at −80 °C immediately after resection. Six normal tissue samples, obtained from six healthy dogs, were used as controls.

Cell lines and culture

Human breast cancer cell line MCF-7 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). This cell line, which is demonstrated to express LAT1 and LAT2 [16,17], was used as a positive control. Canine MM cell lines CMeC-1, CMeC-2, LMeC, PuMeC and KMeC [18,19] were gifted from Dr. Nobuo Sasaki of Tokyo University. All cells were cultured under air with 5% CO_2 at 37 °C in RPMI1640 supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 50 IU/ml penicillin and 50 μg/ml streptomycin.

RNA isolation, RT-PCR and Western blotting

Total RNA extraction, RT-PCR and Western blot were performed according to our previously reported protocols [19]. Specific primers for RT-PCR were designated based on dog *LAT1*, *LAT2* and *ribosomal protein 19* (*RP19*) mRNA sequences obtained from the GenBank database. *RP19*, a housekeeping gene, was used as an internal control. Specific antibodies from rabbit against LAT1 (Cell Signaling Technology, USA) and LAT2 (Abcam, Cambridge, UK) as the first antibodies along with horseradish-peroxidase-labeled goat anti-rabbit IgG as the secondary antibody (Abcam, Cambridge, UK) were used for Western blotting. Detailed information on the primers and cycling conditions is shown in Table 1.

Quantitative analysis by real-time RT-PCR

Quantitative analysis of mRNA expression levels was performed according to our previously reported protocol [20] and relative expression levels (REL) of *LAT1*or*LAT2* mRNA were obtained by normalizing the cDNA numbers of*LAT1*or *LAT2* to those of *RP19*.

Inhibitory studies of amino-acid uptake

Inhibitory studies of amino-acid uptake were performed using [^{3}H]L-leucine (Nihon Medi-Physics Co. Ltd., Tokyo, Japan), one of the substrates of system L amino acid transporters, with or without one of the LAT1 inhibitors BCH (2-amino-2-norbornane-carboxylic acids) or melphalan (LPM) [11]. Because the uptake of amino acids by system L amino acid transporters is not dependent on sodium [1,2,6,21], these experiments were performed in sodium-free uptake solution (125 mM choline-Cl, 4.8 m M KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, 1.2 mM KH_2PO_4, 5.6 mM glucose, pH 7.4). After cells maintained at 37 °C in 5% CO_2 were seeded on 24-well plates (1×10^5 cells/well), uptake measurements were performed when the cells reached approximately 85–95% confluence. After removal of medium, cells were washed three times with sodium-free uptake solution and then pre-incubated in the solution for 10 min at 37 °C. The solution was replaced by new uptake solution (500 μl/well) containing 1-μM [^{3}H]L-leucine with BCH (0, 1, 3, 10, 30 and 100 μM) or LPM (0, 0.1, 0.3, 1, 3 and 10 μM). Because uptake of [^{3}H]L-leucine by system L amino acid transporters is time dependent and exhibits a linear dependence on incubation time up to 1 min [7,22], uptake incubation was performed for 1 min. Uptake incubation was terminated by removing the uptake solution followed by washing three times with ice-cold uptake solution. Cells were then solubilized with 0.1 N NaOH, and radioactivity was counted by liquid scintillation spectrometry (LSC-6000B, Hitachi Aloka Medical Ltd., Tokyo, Japan). [^{3}H]L-Leucine uptake was expressed as a percentage of control radioactivity counts obtained in the absence of BCH or LPM. The IC_{50} values of BCH and LPM based on amino-acid uptake were calculated.

Inhibitory studies of cell growth (WST-8 assay)

Inhibitory studies of cell growth were performed with or without LAT1 inhibitors BCH or LPM. Cells were seeded on 96-well plates (5×10^3 cells/well) and then incubated in phenol red-free RPMI1640, which was supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum, for 24 h. After the medium was changed, the cells were incubated for 48 h in the phenol red-free RPMI1640 (100 μl/well) with BCH (0.1, 0.3, 1, 3, 10, 30 and 100 mM) or LPM (1, 3, 10, 30, 100, 300 and 1000 μM). Cell growth activity was measured with the WST-8 assay kit (DOJIN, Kumamoto, Japan), as described in a previous report [23]. Growth activity was expressed as a percentage of control absorbance values obtained in the absence of BCH or LPM. The IC_{50} values of BCH and LPM based on cellular growth activity were calculated.

Table 1
Specific primers and cycling conditions for PCR.

Gene	Nucleotide sequence (5′ to 3′)	Amplicons (bp)	Conditions	Accession No.
LAT1	F: CTGGATCGAGCTGCTCATCATC R: ACATCACCCTTCCCGATCTGG	273	95 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s	XM_845083 (dog) AF104032 (human)
LAT2	F: TTCCTGACTTCCTTCGTGCC R: CGTGGGTCTTCATTCTGGCT	233	95 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s	XM_540898 (dog) AF171669 (human)
RP19	F: CCTTCCTCAAAAAGTCTGCG R: GTTCTCATCGTAGGGAGC	95	95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s	XM_538673 (dog) NM_001022 (human)

F, forward; R, reverse; bp, base pair.

Combination use of LAT1 inhibitors with anti-cancer drugs

Inhibitory effects of various anti-cancer drugs on cellular growth activities were examined in the absence or presence of LAT1 inhibitors. Cells were incubated for 48 h with a range of doses of carboplatin (0, 0.1, 0.3, 1, 3, 10, 30 and 100 μM), cyclophosphamide (0, 0.1, 0.3, 1, 3, 10, 30 and 100 mM), dacarbazine (0, 0.1, 0.3, 1, 3, 10, 30 and 100 μM), doxorubicin (0, 0.1, 0.3, 1, 3, 10, 30 and 100 μM), mitoxantrone (0, 0.1, 0.3, 1, 3, 10, 30 and 100 μM), nimustine(0, 0.1, 0.3, 1, 3, 10, 30 and 100 μM), vinblastine (0, 0.1, 0.3, 1, 3, 10, 30 and 100 μM) or vincristine (0, 0.1, 0.3, 1, 3, 10, 30 and 100 μM) in the absence or presence of BCH or LPM at IC_{50} concentrations (BCH: 43 mM, LPM: 1.7 μM), obtained in the inhibitory cellular growth experiments described above. The IC_{50} values of anti-cancer drugs based on cell growth activity were calculated.

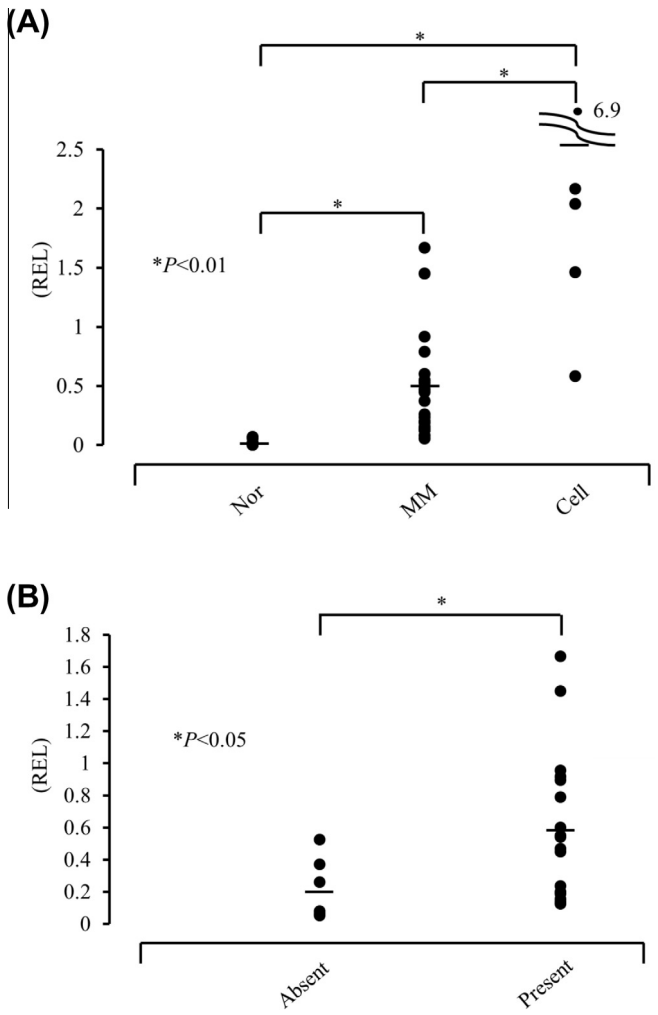

Fig. 1. Quantitative analysis of LAT1 mRNA expression. After LAT1 and RP19 mRNA levels were quantitatively analyzed by real-time RT-PCR, relative expression levels (REL) of LAT1 were calculated by normalizing the LAT1 mRNA levels to RP19 levels. Each point represents an individual LAT1 REL. Horizontal lines represent the mean for each group. (A) REL among normal tissues (Nor, $n = 48$), MM ($n = 25$) and established tumor cell lines (Cell, $n = 5$) were compared. The expression levels in MM and cell lines were significantly ($P < 0.01$) higher than those in normal tissues. (B) REL between MM tissues from patients with (Present, $n = 20$) and without (Absent, $n = 5$) distant metastasis were compared. The expression levels in MM tissues from patients with distant metastasis were significantly ($P < 0.05$) higher than in those without distant metastasis. The Mann–Whitney U test was used to analyze the differences in mRNA expression levels with $P < 0.05$ (2-sided) considered significant. Data analyses were carried out with Excel Toukei 2010 (SSRI, Tokyo, Japan).

Results

Comparative studies among normal tissues, MM and cell lines

To understand the significance of LAT1 expression in MM, *LAT1* mRNA expression was quantitatively compared among normal tissues, MM tissues and cell lines established from MM dogs (Fig. 1A). The expression levels of *LAT1* mRNA in MM (0.45 ± 0.43) and in the cell lines (2.6 ± 2.4) were significantly ($P < 0.01$) higher than those in normal tissues (below 0.01), suggesting an important role for LAT1 in MM. Because the presence of distant metastasis to the lung and lymph node is known as a significant factor defining prognosis in MM [24,25], we divided the MM patients into two groups, "absent" or "present," depending on the presence or absence of distant metastasis. Interestingly, expression in the present group (0.56 ± 0.45) was significantly ($P < 0.05$) higher than in the absent group (0.22 ± 0.19) (Fig. 1B).

LAT1 and LAT2 expression in cell lines

To compare LAT1 and LAT2 expression in five canine MM cell lines, CMeC-1, CMeC-2, LMeC, PuMeC and KMeC, Western blotting and conventional RT-PCR were performed (Fig. 2). LAT1 protein expression was found in all of the five cells while LAT2 was found only in KMeC. Quantitative analysis of mRNA showed REL of LAT1 in CMeC-1 (REL: 1.4) and KMeC (REL: 0.6) to be within the expression range observed in MM tissues from canine patients (REL: 0.05∼1.6). The expression level of LAT2 was very low (REL: 3.0×10^{-7}) in CMeC-1. Thus, because CMeC-1 has an *LAT1* mRNA expression level similar to levels observed in patient tissues and also has a very low level of LAT2 expression, CMeC-1 was chosen as a representative cell line for modeling MM tissues and for functional analysis of LAT1 in subsequent experiments.

Inhibitory studies of amino-acid uptake in CMeC-1

To assess LAT1 function as an amino acid transporter, selective inhibitors for LAT1, BCH and LPM, were chosen for functional analysis in CMeC-1. [^{3}H]L-Leucine (1 μM) uptake was measured in the presence of BCH (0–100 μM) or LPM (0–10 μM) (Fig. 3). BCH and LPM inhibited [^{3}H]L-leucine uptake in a concentration-dependent manner with IC_{50} values of 14 ± 2.1 μM and 1.0 ± 0.04 μM, respectively. These results show that LAT1 expressed in CMeC-1 functions as an amino acid transporter. The IC_{50} value of LPM was close to that of BCH.

Inhibitory experiments of cell growth in CMeC-1

To analyze the effect of BCH or LPM on cell growth, a WST-8 assay was performed on CMeC-1 treated with BCH (0–100 mM) or LPM (0–1 mM) (Fig. 4). BCH and LPM inhibited cell growth of CMeC-1 in a dose-dependent manner. IC_{50} values for BCH or LPM were 43 ± 3.9 mM and 1.7 ± 0.19 μM, respectively. These results show that growth activity is reduced by inhibition of LAT1 function. The IC_{50} value of LPM was remarkably low compared to that of BCH.

Combination use of LAT1 inhibitors with anti-cancer drugs in CMeC-1

To examine the cumulative effects of LAT1 inhibitors on cell growth when used with various anti-cancer drugs, WST-8 assays were performed on CMeC-1 treated with carboplatin (0–100 μM), cyclophosphamide (0–100 mM), dacarbazine (0–100 μM), doxorubicin (0–100 μM), mitoxantrone (0–100 μM), nimustine (0–100 μM), vinblastine (0–100 μM) or vincristine (0–100 μM) in the

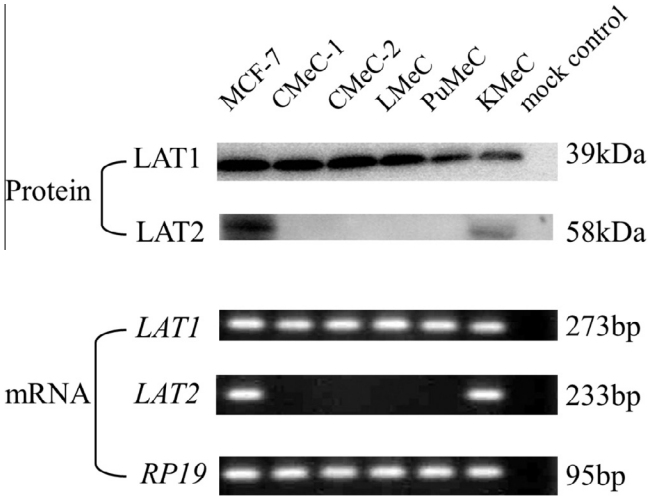

Fig. 2. LAT1 and LAT2 expression in canine MM cell lines. LAT1 and LAT2 expression were analyzed in five canine MM cell lines, CMeC-1, CMeC-2, LMeC, PuMeC and KMeC by Western blotting and conventional RT-PCR. LAT1 protein and mRNA expression were detected in all cell lines examined while LAT2 protein and mRNA expression were found only in KMeC. RP19 mRNA expression was used as an internal control. Human MCF-7 was used as a positive control for the detection of LAT1 and LAT2 expression.

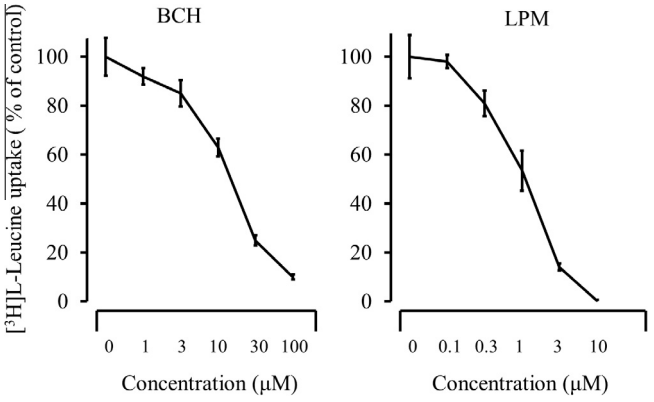

Fig. 3. Concentration-dependent inhibition of [^{3}H]L-leucine uptake by BCH or LPM in CMeC-1 cells [^{3}H]L-Leucine (1 μM) uptake by CMeC-1, measured in the presence of BCH or LPM at various concentrations, is expressed as percent of the control values obtained in the absence of BCH or LPM. Each data point shown is the mean ± SD for three experiments. IC_{50} values for BCH or LPM are 14 ± 2.1 μM and 1.0 ± 0.04 μM, respectively. Incubation was performed in sodium-free uptake solution (free of fetal bovine serum and any nutrient amino-acids) for 1 min.

presence or absence of BCH or LPM at IC_{50} concentrations (Table 2). When administered with one of the LAT1 inhibitors, the IC_{50} values of these anti-cancer drugs were significantly decreased, demonstrating that LAT1 inhibitors enhance the inhibitory activities for cell growth produced by conventional anti-cancer drugs.

Discussion

Canine MM is, as it is in humans, a malignant tumor with aggressive behaviors that invade surrounding tissues and metastasize to regional lymph nodes and lungs, and is believed to be an ideal model of human MM for clinical and therapeutic studies because of the similarities in their biological and clinical behaviors. Because the natural occurrence of canine MM is much higher than human MM and the survival period for dogs with MM is very short compared to human patients [14,15], the dog model is advantageous, allowing us to gather more case data within a shorter time with shorter clinical follow-up periods.

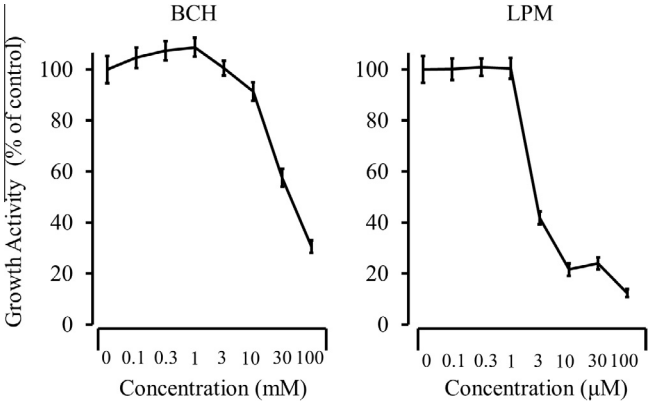

Fig. 4. Inhibition of cell growth activity by BCH or LPM in CMeC-1 cells. Dose-dependentinhibition of cell growth by BCH or LPM was assessed. Cell growth activities, measured using the WST-8 assay in the presence of BCH or LPM at various concentrations, are expressed as percent of the control values obtained in the absence of BCH or LPM. Each data point shown is the mean ± SD for five experiments. IC_{50} values of BCH or LPM are 43 ± 3.9 mM and 1.7 ± 0.19 μM, respectively. Incubation was performed in RPMI1640 supplemented with 2 mM L-glutamine and 10% fetal bovine serum for 24 h.

In humans, LAT1 is expressed preferentially in wide array of tumors but not in normal tissues [8,9,26]. We and others have demonstrated that various canine tumors including mammary gland tumors, thyroid tumors and hemangiopericytomas express LAT1 at high levels compared to normal tissues [20,27], as is also found in human patients [8,9,26]. These coincidental results suggest that LAT1 may play a crucial role in both human and dog tumors. Because no extensive clinical study on LAT1 expression has ever been performed in human spontaneous MM patients, it is unclear whether the up-regulation observed in many other tumors also occurs in human MM. However, based on the facts that (1) LAT1 is expressed highly in dogs with spontaneous MM, and (2) high expression of LAT1 is detected in many naturally occurring tumors and this expression pattern in tumors is found coincidentally in both dogs and humans, it is very likely that LAT is up-regulated in human MM patients. This notion may be supported by a report demonstrating up-regulation of LAT1 in a human cell line derived from a human MM patient [1]. The study of human MM tissues, which is relevant to this research, is eagerly anticipated.

It is reported that the expression levels of LAT1 in human colon cancers, breast cancers, head and neck cancers, genital cancers and soft-tissue sarcomas from patients with distant metastasis are higher than those without metastasis [26], suggesting a role for LAT1 in the biological behavior of tumors. Together with our finding that LAT1 was more up-regulated in dogs with distant metastasis than in those with no metastasis, LAT1 expression could be used as a biological marker for predicting MM behavior in human medicine.

Through inhibitory studies we demonstrated that the LAT1 inhibitors BCH and LPM suppress amino acid uptake as well as cell growth. Although BCH has been reported to inhibit not only LAT1 but also LAT2 [10], the growth inhibitory activity of BCH occurs only through LAT1 inhibition in MM cells, with faint expression of LAT2. Furthermore, we observed that the growth inhibition IC_{50} value of the LAT1-selective inhibitor LPM in MM cells is orders of magnitude lower (1/25,000) than that of BCH, whereas the uptake inhibition IC_{50} values of the two compounds are similar. These findings suggest that the inhibitory effect on cell growth by LPM depends on something other than uptake inhibition through LAT1. Considering that LPM has dual activity as a LAT1-selective inhibitor and alkylating agent, its inhibitory effect on cell growth

Table 2
IC_{50} of various anti-cancer drugs.

Anti-cancer drugs	Single use	Combination with BCH	Combination with LPM
carboplatin (μM)	1.3 ± 0.15	0.66 ± 0.12*	0.58 ± 0.09*
cyclophosphamide (mM)	9.7 ± 2.7	5.3 ± 0.74*	5.8 ± 0.91*
dacarbazine (μM)	10 ± 1.3	6.3 ± 2.0*	5.9 ± 1.1*
doxorubicin (μM)	3.5 ± 0.33	1.5 ± 0.12*	1.6 ± 0.13*
mitoxantrone (μM)	1.8 ± 0.24	0.66 ± 0.07*	0.63 ± 0.08*
nimustine (μM)	8.9 ± 2.2	3.9 ± 0.65*	3.3 ± 0.67*
vinblastine (μM)	52 ± 5.1	20 ± 2.4*	17 ± 2.8*
vincristine (μM)	62 ± 0.14	18 ± 3.4*	19 ± 4.4*

* $P < 0.05$ (versus single use).

likely results from alkylation. Because clinical use of BCH could cause substantial side effects through the suppression of LAT2, which is important for maintaining fundamental cellular activities in normal organs, LPM could be a suitable chemotherapeutic alternative for treating MM that expresses LAT1. In order for LPM to cause growth inhibition through alkylation, it is essential that cells incorporate LPM through the activity of LAT1 because alkylation target sites are intracellular nucleotides and proteins. This notion is supported by a study demonstrating that tumor cells with LAT1 and CD98 down regulation showed LPM resistance [28].

Our finding that combination use of LPM with conventional anti-cancer drugs decreased the IC_{50} values of the anti-cancer drugs shows that LPM is effective for enhancing their cytotoxic effects by inhibiting LAT1 function. Although various general chemotherapy protocols are indicated for treatment of human MM patients in an advanced stage, no effective protocol with satisfactory outcomes is available at present [13]. Our results show that a protocol that integrates LAT1 inhibitors may improve chemotherapeutic outcomes. Additionally, this study may point the way to the use of LAT1 inhibitors in combination with immunotherapy, such as dendritic cell-based vaccination therapy, to which considerable attention has been paid recently as a novel approach to the treatment of MM [29]. LAT1 inhibitors do not affect the immune system because lymphocytes and macrophages, which play a central role in immune response, do not express LAT1.

In conclusion, this is the first report showing the potential for using LAT1 inhibitors as an option in MM therapy. A new combination protocol that includes LPM could be useful for treating human MM expressing LAT1.

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