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Anti-Tumour Effects of High Affinity L-type Amino Acid Transporter1 Inhibitors on Human Pancreatic Adencarcinoma Cells

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Abstract

Background: a system L transporter L-type amino acid transporter 1 (LAT1) is upregulated to support tumor cell growth in malignant tumor. **Objective:** The purpose of the present study was to investigate the growth inhibition and [14C] L-leucine transport in human pancreatic adenocarcinoma cells MAIPaCa-2 and BXPC3. **Methodology:** This animal study was carried out in Japan. The in vitro growth inhibition study was performed by using KYT0351 and KYT0353 which inhibited the tumor cell growth in dose dependent manner and uptake of [14C] L-leucine by MIAPaCa-2 and BXPC3 cells was Na+- independent and was strongly inhibited by KYT0351 and KYT0353. The in vivo tumor growth inhibition was also carried out by intra tumor injection of KYT0351 and KYT0353 at the concentration of 2.6mM of each on both the MIAPaCa-2 and BXPC3 nude mice tumor. **Result:** In a subsequent survival study with the intra peritoneal injection of ascites mice model, control mice had a mean life span of 20 ± 4.30 days and 21 ± 5 days in MIAPaCa-2 and BXPC3 cells respectively, whereas the intraperitoneal injection of 10mg/kg twice daily of KYT0351 and KYT0353 group had improved survival (mean life span 28.4 ± 8.5 and 34.4 ± 9.86 days, 26 ± 4.52 and 31.8 ± 7.62 days respectively in MIAPaCa-2 and BXPC3 cells). Kaplan-Meier survival data of nude mice treated with KYT0351 and KYT0353 were significant. To study the mechanism of growth inhibition we investigated the MIB-1 proliferation assay and TUNEL assay. Significantly less MIB-1 staining and more apoptotic nuclei was detected in tumors treated with KYT0351 and KYT0353 in both MIAPaCa-2 and BXPC3 cells compared with saline treated group. **Conclusion:** In conclusion both the KYT0351 and KYT0353 is a potent LAT1-specific inhibitor and LAT1 could be one of the molecular target in pancreatic adenocarcinoma therapy. [Journal of National Institute of Neurosciences Bangladesh, 2016;2(2): 55-68]

Keywords: L- type amino acid transporter (LAT1); pancreatic adenocarcinoma; KYT0351 and KYT0353

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Introduction

An increase in the transport of amino acid is observed in the state of cell proliferation and tumour cell growth for the protein biosynthesis and energy requirements[1]. The higher demands of amino acids and energy requirements have been identified in the nutrient-poor

tumor microenvironment[2] which is mediated by specific transporters systems. The increase in number of these transporters has been found to correlate with increased transport of amino acids and, in turn, with the growth rate of these cells[2-7]. The transport of large neutral amino acids, in particular those with branched and aromatic light chains, has been attributed to system L. In malignant tumours, the first isoform of system L, L-type amino acid transporter 1 (LAT1) is up regulated to support tumour cell growth[8-10]. LAT1 is highly expressed in culture cells as well as in malignant tumours, presumably to support their continuous growth[11-16]. Tumour antigen 1 (TA1) which is the partial sequence of LAT1 has been identified and shown that it was over expressed in rat hepatocellular carcinoma, human colon cancer and other primary neoplasias[17-18]. There is also transient intermediate upregulation of TA1 (LAT1) was observed in liver regeneration after partial hepatectomy and toxic injury[19]. Adaptive regulation of messages for LAT1 but not for 4F2hc was observed in response to arginine level in normal hepatic cells[20] and it is suggested that upregulation of LAT1 or functional 4F2 complex contribute to neoplastic phenotype as loss of LAT1 regulation with corresponding increased system L transport was observed that associated with malignant progression[21]. Involvement of 4F2 light chain in tumour progression is strongly suggested by a recent study, which showed that upregulation of the 4F2 complex, but not the 4F2 heavy chain alone, in Balb3T3 cells resulted in tumorigenicity in nude mice[22].

Pancreatic adenocarcinoma is the fourth and fifth leading cause of cancer in men and women, respectively, with a dismal 5-years survival rate of <5%[9-10]. Pancreatic cancer is difficult to detect, resistant to treatment, and is usually discovered after it has metastasized. Nearly every person who develops pancreatic cancer will die from it, the majority within the first year of diagnosis[2]. Although surgical resection is the most effective treatment against pancreatic cancer, the outcome has been unfavorable and also adjuvant chemotherapy and radiation therapy do not contribute a significant impact on improving disease survival[11-12]. Therefore, the development of new approach, new molecular target and therapy is important rationale in pancreatic cancer research. Many factors contribute to neoplastic transformation and malignant progression including expression of oncogene, loss of tumor suppressors, loss of cell-cycle components control, angiogenic and cell adhesion factors modulation etc. There is growing body evidence which suggest the overexpression of amino acid transporter is also being a factor in transformation and carcinogenesis. Several clinical investigations providing evidence the overexpression of LAT1 and its role in amino acid uptake, tumor proliferation and biological aggressiveness. To date, there is no report concerning the system L, LAT1 overexpression in pancreatic carcinoma and antitumor efficacy of LAT1 inhibitor.

With respect to TOR signalling many studied had focused on the system L substrate, leucine which is much sensitive than other amino acid like valine and isoleucine. It is shown that system L transporter is upregulated by activation of the mTOR pathway providing an increased capacity for essential amino acid supply to support cell growth[23]. It is shown that competitive inhibitors of amino acid transport inhibit amino acid induced activation of mTOR in human Jurkat cells[24] and also in bladder carcinoma, oral squamous cell carcinoma and colon cancer, BCH inhibited the tumour cell growth in a dose dependent manner[17,25]. So, the importance of system L amino acid transporter is not merely limited to transporting function but involve in nutrient signalling, malignant transformation. The highly proliferating malignant cells need continuous supply of sugar and amino acids and existence of specific upregulated amino acid transporter system and using a specific inhibitor could be a molecular target for therapy. In this study, examination of both in vivo and in vitro effect of LAT1 specific inhibitor in two human pancreatic carcinoma cell line MIAPaCa-2 and BXPC3 was performed. The current study was aimed at investigating the importance of LAT1 expression in MIAPaCa-2 and BXPC3 human pancreatic cancer cell growth and effect of selective inhibitor BCH, KYT0351, and KYT0353 on pancreatic cancer cell proliferation and apoptosis.

Methodology

Materials: KYT0351 and KYT0353 is kindly gifted by professor Kani, chairman of pharmacology and toxicology, kyorin university school of medicine. [^{14}C] L-Leucine, was purchased from Perkin Elmer Life Science Inc. (Boston, MA), BCH and Leucine were purchased from Sigma (St Louis,Mo). Anti Human L-type amino acid transporter-1 (LAT1) monoclonal antibody purchased from Transgenic Inc. In situ cell death detection kit, AP purchased from Roche. MIB-1 Antibody purchased from Dako cytomation.

Cell Culture: MIAPaCa-2 and BXPC3 cells were purchased from the Health Science Research Resources Bank, Japan Health Sciences Foundation (Osaka, Japan). The MIAPaCa-2 cells were grown in MEM (life technologies) supplemented with sodium

bicarbonate 1.5g/litre, non-essential amino acid 0.1mM, sodium pyruvate 1mM, and 10% (vol/vol) FBS in a humidified atmosphere of 5% CO_2 – 95% air at 37º C. The cells were grown for 3 days to confluence, trypsinized and centrifuge at 200×g rpm for 5 minutes, and resuspended in culture medium. The cells were collected and seeded on 24 well plates ($1x10^3$cells/ml/well) in the fresh growth medium. BXPC3 cells were maintained in the growth medium RPMI 1640 supplemented with 10% fetal bovine serum. The cells were collected and seeded on 24 well plates ($1x10^3$cells/ml/well) in the fresh growth medium. Animals: 6 weeks old BALB/cAJc1-nu/nu male nude mice were purchased from CLER, Japan. The animal were acclimated to our laboratory for 4days prior to the injection of culture/ tumor originated cells and then randomly assigned to a saline group, HCL , BCH, KYT0351 and KYT0353 group. The animals were housed in individual cages and maintained under controlled conditions of light (12-hr light/dark cycle) and temperature (23 to 25ºc) and allowed to eat ready made sterile food and sterile water.

In vitro cells growth study: MIAPaCa-2 cells were maintained in the growth medium (minimum essential medium) supplemented with 10% fetal bovine serum at 37°C in 5%CO_2.The cells were collected and seeded on 24 well plates ($1x10^3$cells/ml/well) in the fresh growth medium. BXPC3 cells were maintained in the growth medium (RPMI 1640) supplemented with 10% fetal bovine serum. The cells were collected and seeded on 24 well plates ($1x10^3$cells/well) in the fresh growth medium. After 12 hours of seeding cells compounds are added in the medium at a final concentration of BCH 20 mM, KYT 0351 and KYT0353 at 1, 10, 30 and 100 µM. The cells are subjected to count at different time points. The cells were wash with PBS three times and trypsin100 µl was added in each well and incubate 37°C for 5 min. The cells are collected with 1 ml fresh medium and centrifuged. The cells are resuspended with 100 µl medium and 10µl of was picked up in improved Neubauer counting chamber for counting. Four wells of MIAPaCa-2 and BXPC3 cells were used for each data points and each value represents the mean ± SD.

Inhibition study: MIAPaCa-2 cells were maintained in the growth medium (minimum essential medium) supplemented with 10% fetal bovine serum at 370C in 5% CO_2.The cells were collected and seeded on 24 well plates ($1x10^5$cells/well) in the fresh growth medium. BXPC3 cells were maintained in the growth medium (RPMI 1640) supplemented with 10% fetal bovine serum. The cells were collected and seeded on 24 well plates ($1x10^5$cells/well) in the fresh growth medium. The uptake measurements were performed at 48 h after seeding. After removal the medium, the cells were washed three times with the standard uptake solution (125mM choline-Cl, 4.8Mm KCl, 1.3mM $CaCl_2$,1.2 mM $MgSo_4$, 25mM HEEPS, 1.2mM $KH2PO_4$, 5.6 mM Glucose (PH 7.4) and pre incubated for 10 min at 370C. To evaluate the inhibitory effects of KYT0351 and KYT0353 compounds on [^{14}C] L –leucine uptake, cells were incubated in a solution containing [^{14}C] L –leucine in the absence or presence of inhibitors at 37ºC. The uptake of 1µM [^{14}C] L –leucine was measured in the presence or absence of varied concentration of nonlabeled test compounds unless otherwise indicated. Then, medium were replaced by uptake solution containing [^{14}C] L-leucine. The uptake was terminated by removing the uptake solution followed by washing three times with ice cold uptake solution .Then cells were solubilized with 0.1N sodium hydroxide and radioactivity was counted by liquid scintillation spectrometry. The values are expressed as pmol/mg protein/min. To confirm the reproducibility of the results, three separate experiments were performed for each measurement.

Tumor cells implantation and intratumor injection of compounds on the nude mice: MIAPaCa-2 and BXPC3 cultured cells are prepared for subcutaneous injection in nude mice and wait till well visualized tumor are formed. Tumors bearing nude mice were anesthetized with pentobarbital 50 mg/kg intraperitoneally and tumors were removed by separating outer cover and blood vessels. Tumor cells were collected by smashed tumor in nylon mesh and wash by FBS free MEM medium and FBS free RPMI 1640 medium for MIAPaCa-2 and BXPC3 respectably; for several times. The cells are centrifuged at 1000 rpm for 5 min and resuspended in FBS free MEM/RPMI medium. A single injection of tumor cells were injected subcutaneously on the abdominal wall of male nude mice (BALB/cAJc1-nu) at a concentration of 1×10^6 cells in a volume of 0.5ml in FBS free medium at single point. The tumor was allowed to grow 7 days and form visible tumors and all the mice were randomly separated into 4 treatment groups(n=4): Saline group, BCH group, KYT0351 group and KYT0353 group. Injected volume was 100 µ1 with BCH 258 mM, KYT0351 and KYT0353 2.6 mM. After 7 days of the tumor cell implantation the treatment was started by direct intra tumor injections twice daily and tumor volume was measured by using vernier caliper

before injection. The volumes of tumors were calculated by using the formula: as describe else where[23]. The experiment was terminated when the any group of mice showed severe morbidity.

Survival assay: The cells were collected by added trypsin 500 µl in selected plate after wash with PBS and incubate 5 min at 37°C at incubator. 10 ml fresh medium was added and centrifuged at room temperature at 1000 rpm for 5 min. Discard supernatant and cells were resuspended with MEM 10% FBS medium and washed with FBS free medium. The cells were counted and injected intraperitoneally at a concentration of 5x107cells in volume of 1 ml in FBS free MEM on male nude mice (BALB/cAJc1-nu) and observe for 24hrs. The mice were randomly separated into 3 treatment groups (n=5): Saline group, KYT 0351 group and KYT 0353 group. KYT0351 and KYT0353 compound injected intraperitoneally at a dose of 10mg/kg body weight twice a day. Body weight was measured daily before injection of compound and continue the experiment until death of the mice.

Immunohistochemistry: The tumor was excised from nude mice and the specimen were kept in 4% PFA for 12-16 hours and processed for paraffin block and 5µm tissue sections were prepared and mounted on microscope slides. The tissue sections were then dried and dewaxed, rehydrated and autoclaved 1210C for 5 min and treated with 10% H_2O_2 for 10 min to eliminate endogenous peroxidase activity. After rinsing in 0.05M Tris-buffered saline containing 0.1% tween-20, the section were incubated with anti-LAT1monoclonal C-terminus antibody (1: 50) or anti-4F2hc antiserum (1:50) overnight at 40c and the primary antibody was washed away twice with 0.05M TBS. There after, they were treated with eversion (+) HRP anti mouse (Dako cytometry) for 30 min. They were finally developed in 0.02% diaminobendizine (DAB) with 0.02% H2O2. To verify the specificity of immunoreactions by absorption experiments, the tissue were treated with primary antibodies in presence of antigen peptides (200 µg/ml). All the slides were counter stained with haematoxylin for 1 min and extra stain was washed away with circulating tap water for 15 mins. The slides were then transferred through an ascending ethanol series and finally with xylene and then mounted and observe under the microscope.

TUNEL Assay: The tumor was excised from nude mice which were treated by saline, HCL, BCH KYT0351 and KYT0353 and the specimen were kept in 4% PFA for 12-16 hours and processed for paraffin block and 5µm tissue sections were prepared and mounted on microscope slides. To detect apoptotic cells, the TUNEL assay was performed with in situ cell detection kit (Roche) according to the manufacturer instructions. Briefly, sections were dewaxed, rehydrated and incubate 30 min at 37°C with protease. Then the slides were washed three times for 5 mins with PBS and permeabilized the sections by keeping on ice for 2 mins. The sections were incubated in TUNEL-reaction mixture for 60 min at 37°C. After washing slides with PBS three times for 5 min, an anti-fluorescein antibody conjugated with horseradish peroxidase was added and incubate for 30 min at 37°C to detect labeled nucleotides at free 3′-OH DNA ends. The sections were washed with PBS, and the substrate reaction for peroxidase was performed. Sections were then washed in water and counterstained into the haematoxylin solution for 1 min and wash the slides with circulating tap water for 5 min and then mounted and observe under the microscope.

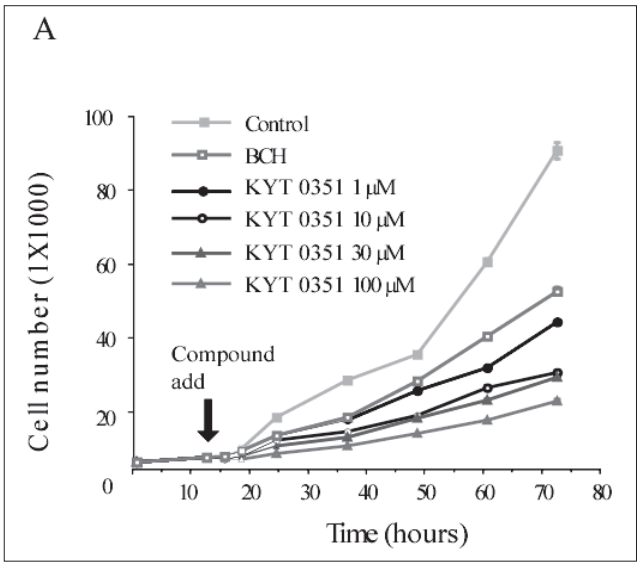
Proliferating assay by MIB-1: The tumor was excised from nude mice which were treated by saline, HCL, BCH KYT0351 and KYT0353 and the specimen were kept in 4% PFA for 12-16 hours and processed for paraffin block and 5µm tissue sections were prepared and mounted on microscope slides. The tissue sections were then dried and dewaxed, rehydrated and autoclaved 121°C for 5 min and treated with 10% H2O2 for 10 min to eliminate endogenous peroxidase activity. After rinsing in 0.05M Tris-buffered saline containing 0.1%tween-20, the section were incubated with anti-KI67 monoclonal antibody (1:50) overnight at 40c and the primary antibody was washed away twice with 0.05M TBS There after, they were treated with eversion (+) HRP anti mouse (Dako cytometry) for 30 min. They were finally developed in 0.02% diaminobendizine (DAB) with 0.02 % H_2O_2. To verify the specificity of immunoreactions by absorption experiments, the tissue were treated with primary antibodies in presence of antigen peptides (200µg/ml). All the slides were counter stained with haematoxylin for 1 min and extra stain was washed away with circulating tap water for 15 mins. The slides were then transferred through an ascending ethanol series and finally with xylene and then mounted and observe under the microscope.

Statistical analyses: A comparison of the survival curves between each treatment and control group was performed by a log-rank test and data expressed as mean ± SD. Statistical differences were determined using students unpair t-test. Differences were considered significant at $p< 0.05$.

Results

In vitro growth inhibitory effect of KYT0351 and KYT0353 compounds on the growth of MIAPaCa-2 and BXPC3 cells: MIAPaCa-2 and BXPC3 cells were seeded at a density of $1x10^3$ cells/ml/well and the cells were grown 12 hrs without any treatment. After 12 hrs, cells medium was replaced by new medium containing BCH and different concentration of KYT compounds. 20mM BCH was used as a positive control and 1-100µM of KYT compounds was used to evaluate the growth inhibitory effects. Cells were counted at 15hrs, 18hrs, 24hrs, 36 hrs, 48hrs, 60hrs and 72 hrs.

Compound KYT0351 (Fig.1A) and KYT0353 (Fig.1B) shows growth inhibitory effect in a dose depending manner in MIAPaCa-2 cells. Similar effects was observed in BxPc3 cells in compound KYT0351 (Fig.1C) and KYT0353 (Fig.1D). BCH is a system L specific inhibitor that shows potent inhibitory effects than control. In contrast to BCH the newly compounds KYT shows more potent effects than the BCH and the inhibitory gradient as KYT0353>KYT0351>BCH. These results suggest that the newly prepared compounds have more potent inhibitory effects than the BCH and specific inhibitor for system L, LAT1.



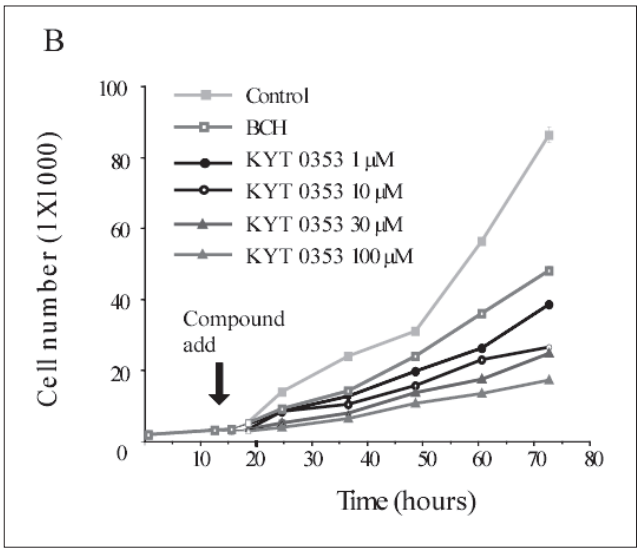
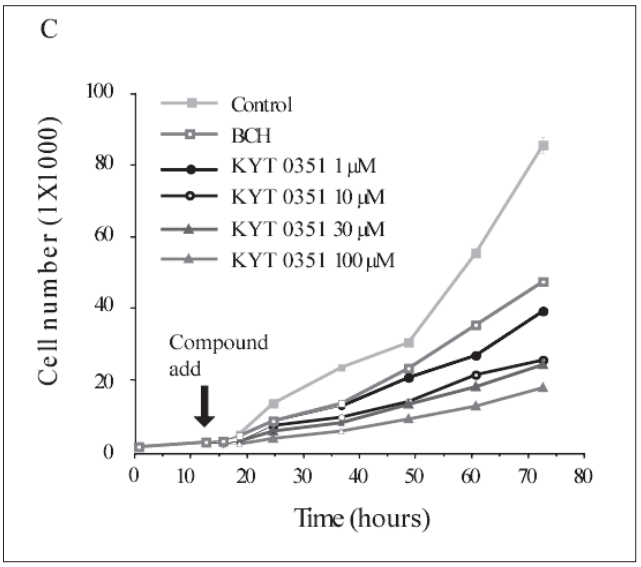


Figure 1: Inhibitory effects of compounds on the cell growth of pancreatic tumor cell lines. Cells were incubated with 20mM BCH and various concentrations of KYT compounds on MIAPaCa-2 pancreatic tumor cell line (A, B) and BxPc-3 pancreatic tumor cell line (C,D). Each value is the mean ±S.D of three different experiments



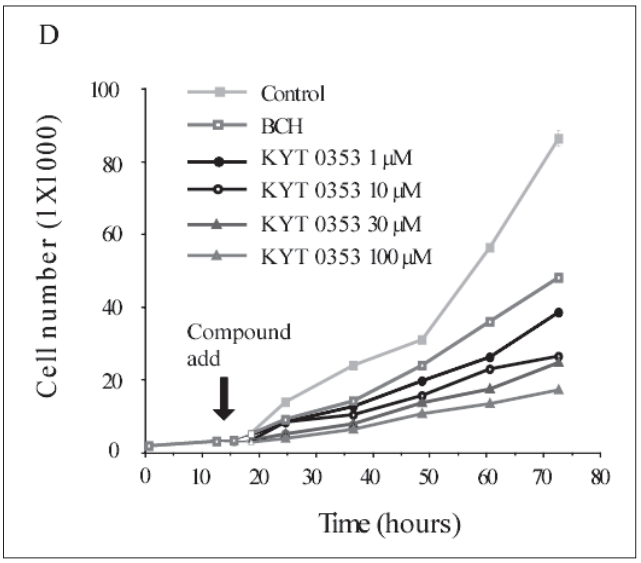
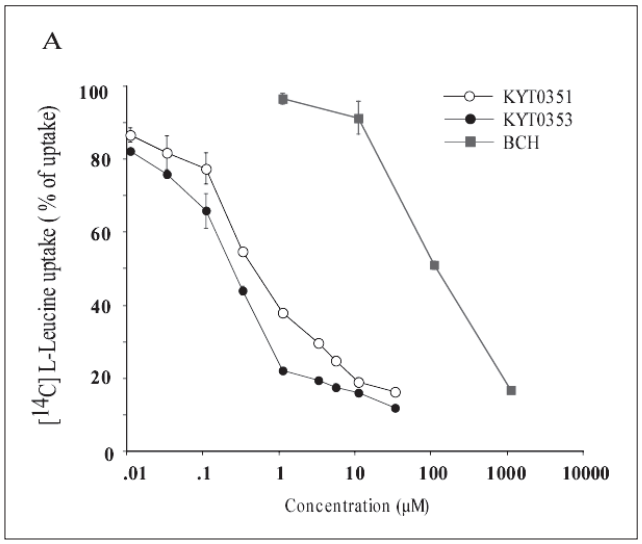
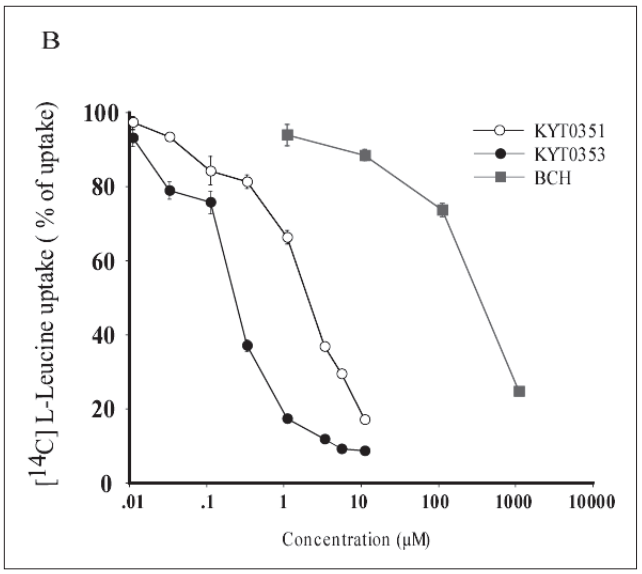


Figure 2: The inhibitory effects of BCH and KYT compounds on the [14C]L-leucine uptake in pancreatic tumor cell lines.(A) The concentration-dependent inhibitory effect of KYT0351 and KYT0353 on [14C]L-leucine uptake in MIAPaCa-2 and (B) The concentration-dependent inhibitory effect of KYT0351 and KYT0353 on [14C]L-leucine uptake in BXPC3 cells. The [14C]L-leucine uptake (1µM) was





measured for 1 min in the Na+- free uptake solution in presence of varied concentration of BCH, KYT0351 and KYT0353 and expressed as a percentage of control L- leucine in absence of BCH, KYT0351 and KYT0353.

Inhibition of [14C] L-leucine uptake in pancreatic tumor cell line MIAPaCa-2 and BXPC3 cells with amino acid related compound BCH and KYT: To evaluate the inhibitory effects of KYT0351 and KYT0353 compounds on [14C] L –leucine uptake, cells were incubated in a solution containing [^{14}C] L –leucine in the absence or presence of inhibitors at 37^{o}C. The uptake of 1μM [^{14}C] L –leucine was measured in the presence or absence of varied concentration of non labeled test compounds BCH, KYT0351 and KYT0353. BCH was used for 1-1000μM and KYT compounds from 0.01-30μM. The uptake of [14C] L- leucine was inhibited in a concentration dependent manner in both cell line MIAPaCa-2 (Fig 2A) and BXPC3 (Fig. 2B) by the test compounds. The IC_{50} value of test compound was tabulated in Table. 1. It shows that the IC_{50} value of BCH is almost similar in both cell lines. In MIAPaCa-2 and BXPC3 pancreatic tumor cell lines KYT 0353 shows almost two times stronger effects than KYT0351 and in compare to BCH the compound KYT0351 shows more than 200 times stronger effects in MIAPaCa-2 and >100times in BXPC3 cell line, whereas KYT0353 shows >300 times stronger inhibitory effects in MIAPaCa-2 and >150 times in BXPC3 tumor cell line. The inhibitory effects on the uptake of [14C] L- leucine of test compound was graded as KYT0353>KYT0351>BCH.

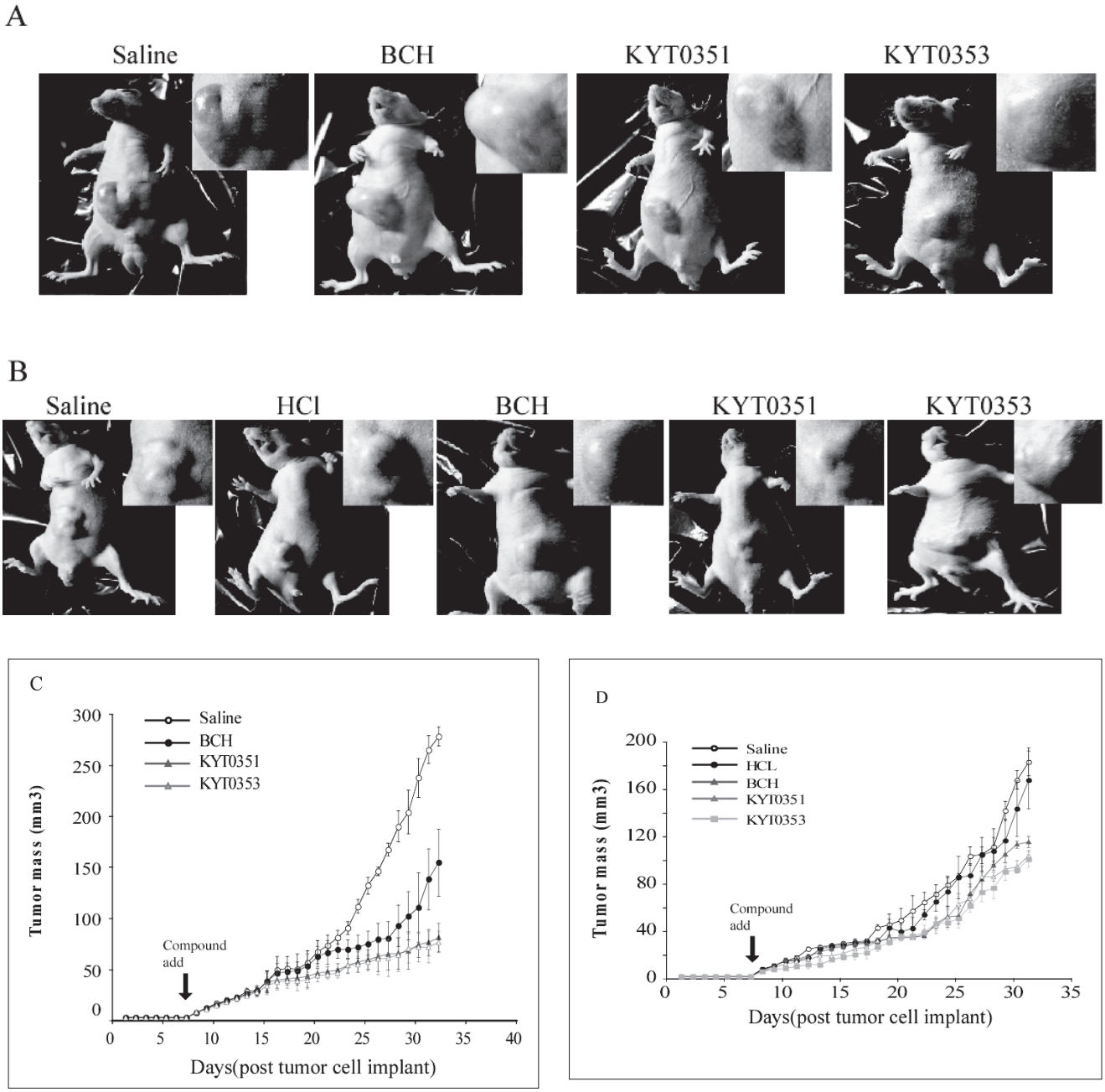
Table 1: IC_{50} of compounds in pancreatic tumor cells

	IC_{50} (μM)	
Compound	**MIAPaCa-2 cell**	**BXPC3 cell**
BCH	97.66 ± 1.45	99.33 ± 2.96
KYT0351	0.43 ± 0.11	0.915 ± 0.05
KYT0353	0.29 ± 0.06	0.578 ± 0.03

Growth inhibition was determined as described in Materials and methods section. IC_{50} values are given as mean value ± S.E of three independent experments.

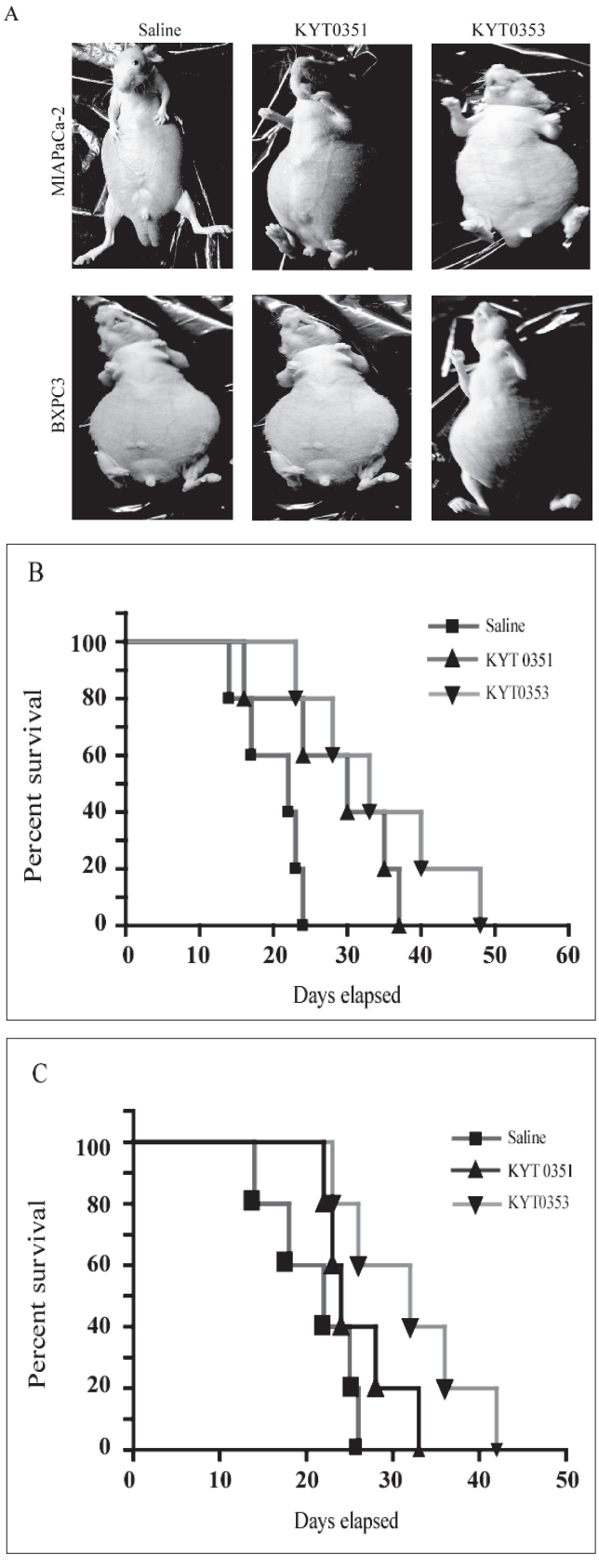
Figure 3: Effect of soluble LAT1-specific inhibitors on growth of MIAPaCa-2 and BXPC3 tumor in nude mouse. 1x 106cells were injected s.c. into nude mice. At day 7 when well visible tumor had formed, animals were treated with BCH (258mM), KYT0351 and KYT0353 (2.6mM) or control vehicle saline and HCl twice daily. The size of the tumor were measured daily and results represent mean ± SD of tumor volume (n= 5) in two independent experiments. (A, C) Visible tumor and effect of compounds on MIAPaCa-2 cell tumor in nude mice. KYTO351 and KYT0353 treatment causes regression in tumor growth. The difference among the treatment and control groups was statistically significant (p= 0.0106 control vs KYT0351 and p> .008 control VS KYT0353. (B, D) Visible tumor and effect of compounds on BXPC3 cell tumor in nude mice. The growth inhibition effect was observed in compound treated groups and the inhibition was statistically significant (p= .0506 in control VS KYT0351 and p> .08 control VS KYT0353).

In vivo effect of soluble LAT1-specific inhibitor on MIAPaCa-2 and BXPC3 cells nude mouse tumor: To investigate the effects of the compounds on the growth of the tumor in an animal model direct intra tumor injection of KYT0351 and KYT0353 was performed starting from day 7 after cells implantation. Figure 3 A & B shows the nude mice bearing MIAPaCa-2 and BXPC3 inducing tumor. The tumor volume was measured daily and the results were shown in fig. 3C&D. In MIAPaCa-2 tumor cell inducing tumor a



significant inhibition of the growth of the tumor was observed in compound treated groups and among the tested compounds KYT compounds shows stronger inhibitory effect than BCH. In BXPC3 cell inducing tumor, we also included HCl in the treatment group as HCl is the solvent for the compound and there is no significant difference in the saline and HCl treated group. But the tested compound shows significant inhibition compare to saline and HCl groups and KYT compounds shows stronger inhibition than BCH. In between the cell lines, MIAPaCa-2 shows stronger inhibition in the entire tested compound than BXPC3 cell line. In MIAPaCa-2 cell line BCH shows ~42% inhibition and KYT shows ~75% inhibition than saline. In compare to BCH, KYT inhibit the tumor growth ~55%. In BXPC3 cell line, BCH shows ~ 39% inhibitions and KYT shows ~50% inhibition than saline. In compare to BCH, KYT inhibit the tumor growth ~18%.

Figure 4: MIAPaCa-2 and BXPC3 cells were implanted by intraperitoneal injection in nude mice. After 24 hours, groups of these mice were treated with injections of either KYT0351or KYT0353 (10mg/kg body weight) twice daily and control vehicle saline. The mice were monitored and the day of death of each was noted. The results in term of host survival are shown in the Kaplan-Meyer plot. (A) Nude mice



treated with compounds. (B) Kaplan-Meyer survival curve of control and treated group in MIAPaCa-2 cell implanted nude mice. (C) Kaplan-Meyer survival curve of control and treated group in BXPC3 cell implanted nude mice

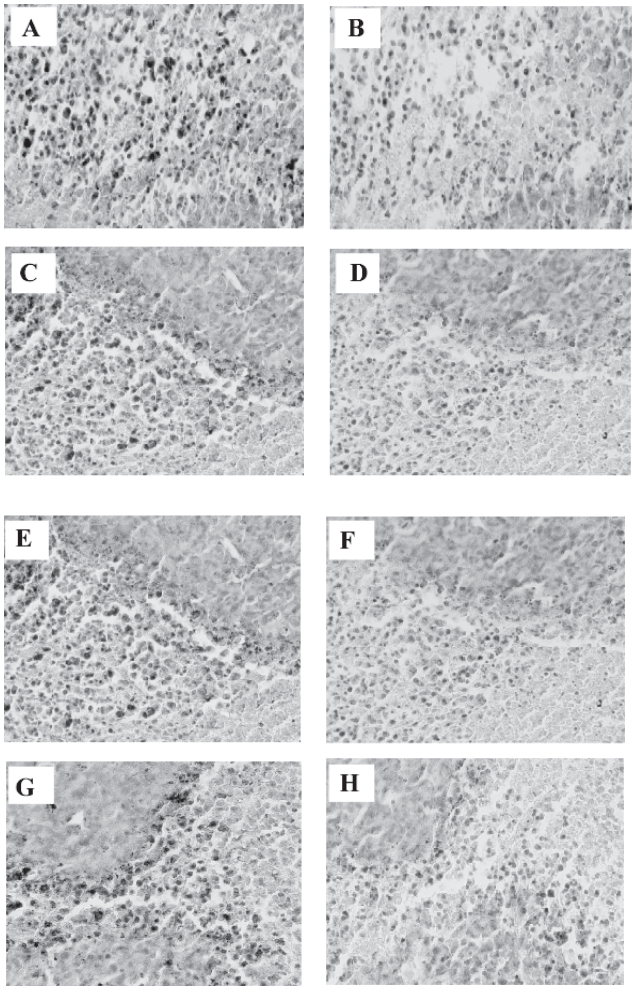
Table 2: Mean survival of nude mice after i.p injection of compounds

Nude mice of MIApaca-2 inducing tumor			
Treatment group	**Number of mice**	**Mean surviva mean± SD**	**Significant level (Log rank test)**
Saline	5	20.0 ± 4.3	
KYT 0351	5	28.4 ± 8.61	*
KYT 0353	5	34.4 ± 9.86	*
Nude mice of BXPC inducing tumor			
Saline	5	21.0 ± 5.00	
KYT 0351	5	26.0 ± 4.52	+
KYT 0353	5	31.8 ± 7.62	*

+p<0.1; *p<0.05 vs saline

Tumor implantation in nude mice and survival assay after treatment with the compound KYT0351 and KYT0353: A single injection of MIAPaCa-2 and BXPC3 culture cells were injected intraperitoneally at a concentration of 5x107cells in volume of 1 ml in FBS free MEM on male nude mice and observe for 24 hours. The mice were randomly separated into 3 treatment groups (n=5) and treat with compounds at the dose of 10mg/kg body weight intraperitoneally twice a day. There is no occurrence of seizures or changes of behavior such as sluggishness or inability to eat in all groups of nude mice (Fig. 4A). The mice were monitored and day of death of each was noted and plotted as Kaplan-Meyer plot (Fig. 4B, C). All of the mice in each group eventually succumbed to their tumor burden, again reflecting the tumorigenicity of MIAPaCa-2 and BXPC3 cell in this orthotopic model and the technical efficiency of the intraperitoneal tumor implantation procedure. Control mice had a mean life span of 20 days for MIAPaCa-2 cell tumor bearing mice and 21 days for BXPC3 cell tumor bearing mice, whereas both the KYT0351 and KYT0353 treated groups had improved survival. In KYT0351 group mean survival for MIAPaCa-2 cell tumor bearing mice is 28.4 days ($p<0.05$) and 26 days for BXPC3 cell tumor bearing mice ($p<0.1$), whereas in KYT0353 group mean survival for MIAPaCa-2 cell tumor bearing mice is 34.4 days ($p<0.05$) and 31.8 days for BXPC3 cell tumor bearing mice ($p<0.05$) (Table. 2). Compound treated groups had a higher mean survival in MIAPaCa-2 cell tumor bearing nude mice and compound KYT0353 had a higher survival rate than compound KYT0351.

Figure 5: Expression of LAT1 and 4F2hc on immunostaining in tumor produced in nude mice with MAIPaCa-2 and BXPC3 cells. (A) LAT1 expressed in MAIPaCA-2 tumor cell, (B) absorption experiments of LAT1 in MAIPaCA-2



tumor cells, (C) LAT1 expressed in BXPC3 tumor cell (D) absorption experiments of LAT1 in BXPC3 tumor cell (E) 4F2hc expression in MIAPaCa-2 cells tumor, (F) absorption experiments of 4F2hc in MAIPaCA-2 cell tumor, (G) 4F2hc expressed in BXPC3 tumor cell, (H) absorption experiments of 4F2hc in BXPC3 cell tumor. Magnification x 200.

Expression of LAT1 and 4F2hc in tumor isolated from nude mice implanted with MIAPaCa-2 and BXPC3 cell: To detect the LAT1 and 4F2hc over expression in pancreatic adenocarcinoma cells, we performed the immunohistochemical analyses by using the tumor tissue produce by MIAPaCa-2 and BXPC3 cells. LAT1 and 4F2hc immunoreactivity was observed in all the tumor specimens. Strong diffuse LAT1 immunostaining was observed in tumor cells (Fig. 5A, C). Strong diffuse 4F2hc immunostaining also was observed in both tumor cells.(Fig. 5 E, G). The absorption experiments in which tissue sections were treated with primary antibodies in the presence of antigen peptide the immunostaining was drastically decreased, confirming the specificity of the immunoreactions (Fig. 5 B, D, F & H).

Figure 6: MIB-1 assay of subcutaneous tumor tissue specimens from nude mice. Tissue samples of nude mouse were collected after four weeks of treatment with saline, BCH or KYT0351 and KYT0353 and processed for KI-67 immunostaining. Representative KI-67 sections were counterstained with haematoxylin for each treatment option. Fig.6A- 6E is the immunostaining of tumor derived from MIAPaCa-2 cells, where (A) saline, (B) HCL, (C) BCH, (D) KYT0351, and (E) KYT0353. Fig.6F- 6J are the immunostaining of tumor derived from BXPC3 cells, where (F) saline, (G) HCL, (H) BCH, (I) KYT0351, and (J) KYT0353. Significantly less staining of KI-67 was detected in tumor treated with KYT0351 and KYT0353 compounds.

Intra-tumor injections of KYT compounds inhibited proliferation and induced apoptosis in both cell lines: To investigate the potential mechanisms by which KYT0351 and KYT0353 might inhibit tumor growth, we sampled tumor from representative animals from the

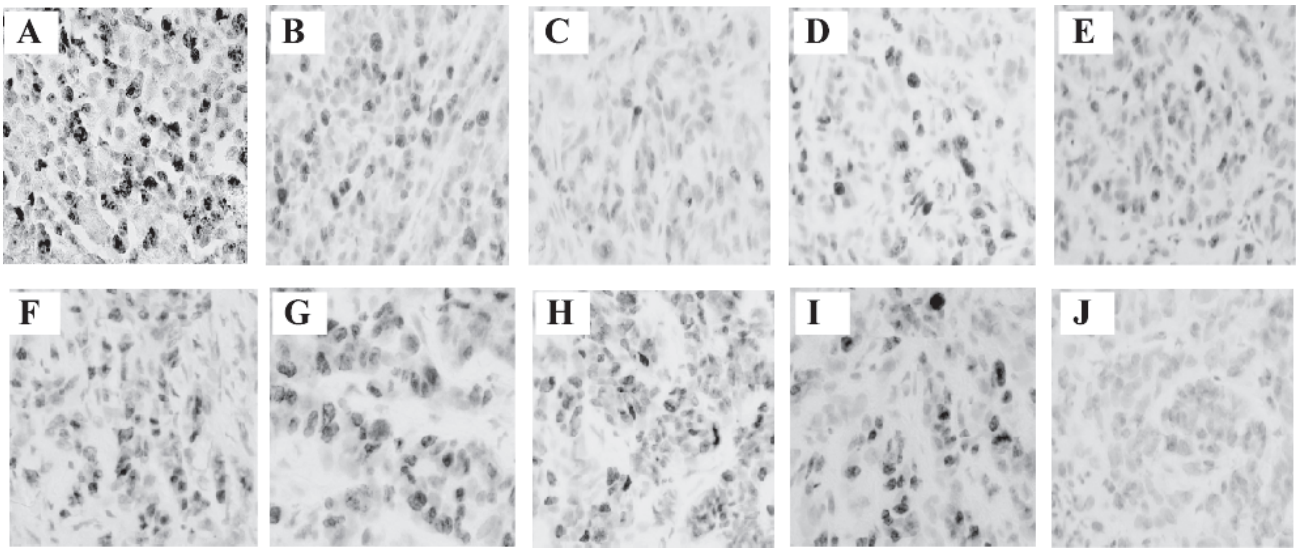


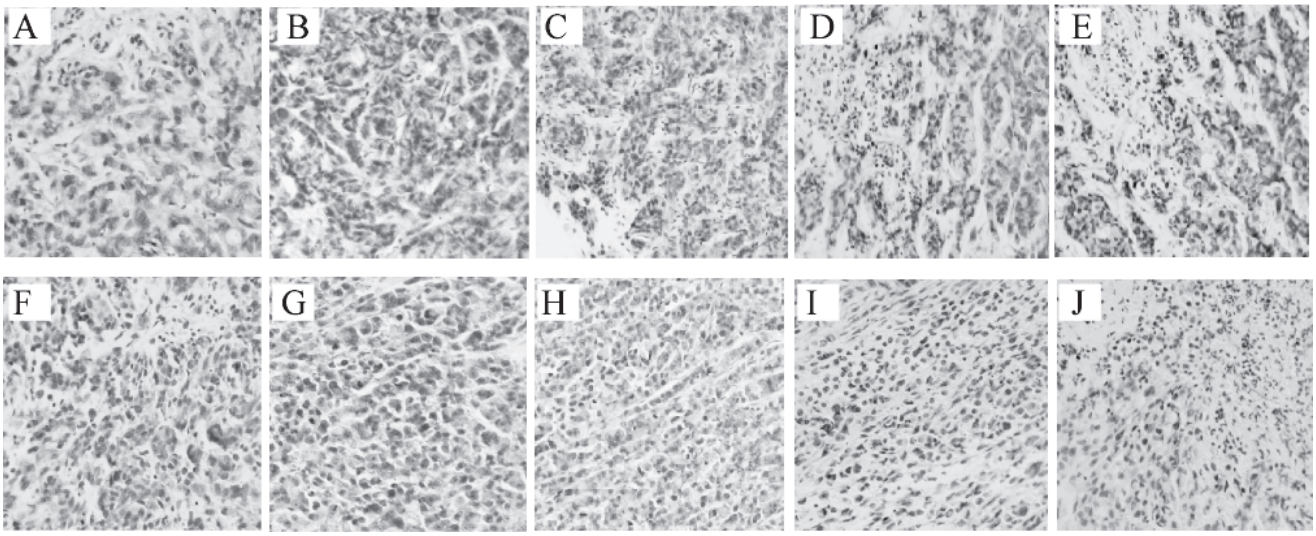
Table 3: MIB-1 labelling index in nude mice tumor tissues inducing by pancreatic tumor cell lines

Treatment group	Number of mice	MIApaca-2 inducing tumor tissue		BXPC inducing tumor tissue	
		Mean MIB-1 index mean± SD	**Significant level (p value)**	**Mean MIB-1 index mean± SD**	**Significant level (p value)**
Saline	3	25.02 ± 2.77		28.10 ± 1.26	
HCL	3	26.65 ± 3.14	NS	26.31 ± 2.39	NS
BCH	3	20.25 ± 1.38	*	22.52 ± 1.75	*
KYT 0351	3	18.60 ± 1.34	**	18.31 ± 1.00	**
KYT 0353	3	6.36 ± 0.92	**	14.37 ± 0.81	***

*p<0.05, **p<0.01, ***p<0.001 vs saline

5 treatment groups and tested them for proliferation assay as determined by MIB-1 assay. The representative immune-staining of MIB-1 was shown in fig. 7 and mean of the labeling index was shown in table 3. The mean labeling index of saline and HCl treated group are almost similar with a mean of 25 to 28. But the MIB-1 labeling index in the tumor treated with BCH, KYT0351 and KYT0353 was significantly ($p<0.001$) lower and KYT compounds shows lower mean than the BCH treated group. Among the KYTs, compound KYT0353 had more potent effect than KYT0351. We tested the apoptosis by measuring the presence of fragmented DNA nuclei of treated the tissue using the TUNEL assay (Figure 7). The mean of the three separate experiments were tabulated and shown in table 4. The tumor tissues treated with saline and HCl group shows similar mean about 27 for MIAPaCa-2 and 24 for BXPC3 cell tumor. We detected significantly higher levels of positive apoptotic nuclei in BCH, KYT0351 and KYT 0353 treated group both in MIAPaCa-2 and BXPC3 cells tumor. The apoptotic index of tumor from the animal treated by KYT0351 and KYT0353 were significantly ($p<0.001$) higher than controls and BCH in both MIAPaCa-2 and BXPC3 cells. Thus, the modulation of cell proliferation and induction of apoptosis appear to reduce tumor size in KYT compound treated groups.

Figure 7: TUNEL reaction on subcutaneous tumor specimens from nude mice. Tissue samples of nude mouse were collected after four weeks of treatment with saline, HCl, BCH, KYT0351 and KYT0353 and processed for KI-67 immunostaining. Fig.7A- 7E is the immunostaining of tumor derived from MIAPaCa-2 cells, where (A) saline, (B) HCL, (C) BCH, (D) KYT0351, and (E) KYT0353. Fig.7F- 7J are the immunostaining of tumor derived from BXPC3 cells, where (F) saline, (G) HCL, (H) BCH, (I) KYT0351, and (J) KYT0353. Significantly more apoptotic nuclei were detected in tumor treated with KYT0351 and KYT0353



Discussion

System L operates as an obligatory 1:1 amino acid exchanger which can couple with cellular uptake of essential branched chain and aromatic amino acids with the efflux of cytoplasmic amino acid such as glutamine, suggesting a net influx of essential amino acids[26] and it is speculated that LAT1 expression is up regulated so as to provide cells with essential amino acids for high levels of protein synthesis associated with cell activation and also to support rapid growth or continuous proliferation.

A high level of LAT1 expression was also detected in human tumor cell lines such as stomach signet ring cell carcinoma (KATOIII), malignant melanoma (G-361), lung small-cell carcinoma (RERF-LC-MA) glioma, breast cancer, bladder cancer, and colon cancer[8,11,19,23,26]. 4F2hc heavy chain is required for the trafficking and regulation of the LAT1 to the plasma membrane[27]. 4F2 is an early T-cell activation antigen[28] which causes malignant transformation when over expressed in NIH3T3 cells[29] and also been implicated both in integrin signalling and regulation of cell –substratum interactions[30]. Furthermore, there is considerable evidence for the over expression of both 4F2hc and its associated light chain in tumours30 and leukaemia isolates[31]. LAT1 has been suggested to act as an 'environmental sensor' of amino acid availability[32] and this function of LAT1 clearly may facilitate by association with 4F2hc. In the functional complex of LAT1 and 4F2hc, LAT1 is the catalytic subunit for the transport of the substrate and in this study we targeted the LAT1 with the amino acid related compounds BCH and KYTs both in in vivo using pancreatic adenocarcinoma cell line MIAPaCa-2 and BXPC3 and in vitro using nude mice model. In our study we clearly demonstrated that LAT1 and 4F2hc was overexpressed in pancreatic adenocarcinoma and LAT1 specific inhibitor BCH and KYTs can inhibit the tumor cell growth and [^{14}C] L-leucine uptake in a concentration dependent manner. Furthermore, these finding illustrated that directly injection of the compounds in the tumor significantly inhibits the tumor cell growth accompanied by dramatic inhibition of cell proliferation and increased apoptosis. This is the first report on the pancreatic adenocarcinoma model to show that amino acid transporter LAT1 specific inhibitor can inhibit the tumor growth.

LAT1 also corresponds to an oncofetal antigen, TA1 that is expressed primarily in fetal tissues and cancer cells and identified as a tumor-associated sequence with the oncofetal pattern of expression in rat liver. TA1 immunoreactivity was abundant in human colon cancer in vivo but barely detected in surrounding normal colon tissue, confirming the high level of expression of LAT1 protein in tumor cells[33-36]. Consistent with this observation and others, it was also found that LAT1 and 4F2hc expressed highly in the tumor produced by MIAPaCa-2 and BXPC3 pancreatic tumor cell lines. Though, it could not differentiate the immunoreactivity of both cytosol and plasma membrane but cytoplasmic LAT1 immunoreactivity may represent the intracellular pool of the LAT1 and may correlate with the biological activity of the cells. Campbell et al[13] examined the response of TA1/LAT1 and 4F2hc expression and amino acid transport to arginine availability in a panel of rat hepatic epithelial cell lines differing in GGT-expression, transformation and tumorigenicity. The authors have showed that TA1/LAT1 is constitutively expressed in GGT-positive transformed cells and do form tumour rapidly in the liver. Whereas 4F2hc mRNA level do not vary significantly in these cells, although in general the levels are much higher in transformed versus nontransformed cells[20]. LAT1 is expressed minimally at the plasma membrane in cancer cells, remaining mostly in the Golgi area, and requires 4F2hc to be sorted to the cell surface

Table 4: Apoptotic index in nude mice tumor tissues inducing by pancreatic tumor cell line

Treatment group	Number of mice	MIAPaCa-2 inducing tumor		MIApaca-2 inducing tumor	
		Mean± SD	(P value)	Mean± SD	(P value)
Saline	5	27.26 ± 2.04		24.63 ± 1.02	
HCL	5	27.19 ± 3.06	NS	23.49 ± 1.01	NS
BCH	5	42.45 ± 1.49	**	40.02 ± 2.39	***
KYT 0351	5	51.38 ± 1.49	***	54.42 ± 5.53	***
KYT 0353	5	62.04 ± 2.69	***	67.96 ± 2.06	***

*p<0.05, **p<0.01, ***p<0.001 vs saline

consistent with the finding of rapid degradation signal AUUUA at LAT1[5]. The immunoreactivity of LAT1 in the plasma membrane may represent its function and as that of essential amino acid, LAT1 also accept amino acid analogue BCH which is a nonmetabolizable artificial amino acid and a transportable inhibitor for LAT1. Treatment of the pancreatic cancer cell line MIAPaCa-2 and BXPC3 with BCH and KYTs showed the growth inhibition and also [^{14}C] L-leucine uptake in a concentration dependent manner, providing further evidence that LAT1 plays an important role in the proliferation of human pancreatic cancer cells.

Immunohistochemically determined MIB-1 fractions are clearly associated with some form of cancer[10-15]. Kunaiki et al and Kitamure et al showed that Ki-67 labelling index increased form low grade to high grade AAH to NMBAC, indicating a gain in proliferation activity from premalignant cell to malignant cells. Furthermore Kunaiki et al suggest that proliferating cells underwent an increase in their metabolism with an associated upregulation of LAT1 protein. Using the athymic mice model, we immunostained the MIB-1 in the tissue sections of control vehicle and compound BCH, KYTs treated groups. The mean MIB-1 labelling index reduces by ~20% in BCH treated group, ~28% for MIAPaCa-2 and ~35% for BXPC3 in KYT0351 treated group and ~76% in MIAPaCa-2 and ~50% for BXPC3 in KYT0353 treated group. This result clearly indicated the inhibition of the LAT1 with LAT1 specific inhibitor KYTs dramatically reduces the proliferation of the tumor cell as well as the tumor volume.

Apoptosis, or programmed cell death, is an intrinsic cell suicide mechanism that plays a important role in the development and maintenance of healthy tissues. It is morphologically distinct from necrotic cell death. Deregulation of this cell death pathway occurs in cancer, autoimmune disease, and neurodegenerative disorders. In recent years, emphasis has focused on the importance of apoptosis as the mechanism by which chemotherapy and irradiation kill cancer cells. A reduction in apoptosis favours malignant transformation and proliferation of cancer cells, whereas most chemotherapeutic agents induce apoptosis[37]. The present study shows that treatment of the pancreatic tumor in nude mice with the LAT1 specific inhibitor KYTs inhibit proliferation and induce apoptosis. Athymic mice bearing s.c. transplanted human pancreatic cancer cells have proven to be effective model for studying in vivo effects of anticancer treatments[38-39]. Intratumor injection of the compounds BCH, 256mM and KYTs 2.6mM dramatically inhibited the tumor growth compared with that seen in vehicle treated control animals. There did not appear to be any toxic effects of the compounds at the dose used in these animals. All animals tolerated the treatment very well, and there were no changes in the behaviour. To clarify the potential mechanism involved, the proliferation assay was done with MIB-1 and TUNEL assay. MIB-1 proliferation assay indicates that the mean MIB-1 labelling indices were significantly lower in the KYTs treatment group compared to control groups. In contrast, TUNEL assay indicated the mean apoptosis indices was significantly higher in the KYTs treatment groups compared to control vehicle treated groups. Taken together these result suggest that induction of apoptosis is likely to be responsible for the tumor growth inhibition in these mice.

Kim et al[22] reported the role of LAT1 as a potential therapeutic target in hepatic tumor cells in vitro. Several clinical investigations demonstrated the significant relation of the uptake of radiolabeled amino acid in gliomas and proliferation, biological aggressiveness or histological grading of these tumors and also demonstrated significant longer survival times in patients with cerebral gliomas with low amino acid uptake than in gliomas with high amino acid uptake. Another study on BALB3T3 cells clarified that efficacy of tumour formation is depending on the expression of endogenous heterodimeric 4F2hc and that the light subunit LAT1 is essential for malignant phenotype caused by over expression of 4F2hc[22]. Over expression of LAT1 and 4F2hc is a possible cause of increased tumour cell growth and proliferation. Using T-24 bladder carcinoma cell line Kim et al[22] clearly showed that BCH inhibited the tumour cell growth in a dose dependent manner. A recent study on vascular smooth muscle cells showed that inhibition of LAT1 activity by BCH markedly inhibit the proliferation of vascular smooth muscle cells whereas BCH has no effect on the viability of quiescent smooth muscle cells[40]. This result is consistent with the study in oral squamous cell carcinoma by Kim et al[22] and they also suggest that LAT1 protein can play an important role in the oral squamous epithelial carcinogenesis which is also suggested by colon cancer study[41-44]. Consistent with this, in this study, we also observe the similar effects of BCH in both in vivo and in vitro model. Additionally, we emphasises on the KYTs, which are the newly synthesized amino acid analogues has more

effective inhibitor that specifically inhibit LAT1, an essential amino acid transporter.

Pancreatic cancer continues to represent a significant oncological challenge[45-46]. The difficulty of early diagnosis of pancreatic cancer and the lack of therapeutic options for pancreatic cancer have prompted the search for new treatment approach, molecular target, and therapeutic agents. The surface membrane compartment of the cell is of substantial interest and the cell surface protein that is overexpressed and related to the tumor proliferation, progression and signalling pathway can be utilized for either antibody or drug based therapy. In this heterodimeric complex of system L, it would be possible to generate LAT1- permeable anti-tumour drugs considering the broad substrate selectivity of LAT1 and antibody-based therapy for 4F2hc. Previous work has demonstrated that antibodies to 4F2-like molecules reversibly inhibit bladder cancer and T-lymphoma cell growth in vitro in a dose dependent manner.

Conclusion

In conclusion both the KYT0351 and KYT0353 is a potent LAT1-specific inhibitor and LAT1 could be one of the molecular targets in pancreatic adenocarcinoma therapy. The findings of the overexpression of LAT1 and its heavy chain 4F2hc coupled with the effectiveness of the LAT1 specific inhibitor in blocking proliferation and inducing apoptosis, as revealed by the experiments performed in nude mice subcutaneously implanted with the human pancreatic carcinoma cell line MIAPaCa-2 and BXPC3, providing a promising avenue for therapeutic intervention in this devastating disease.

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