

Inhibition of oligopeptide transporter suppress growth of human pancreatic cancer cells

メタデータ	言語: English 出版者: 公開日: 2017-10-04 キーワード: 作成者: Mitsuoka, Keisuke, Kato, Yukio, Miyoshi, Sosuke, Murakami, Yoshihiro, Hiraiwa, Mariko, Kubo, Yoshiyuki, Nishimura, Shintaro, Tsuji, Akira メールアドレス: 所属:
URL	http://hdl.handle.net/2297/24628

Inhibition of Oligopeptide Transporter Suppress Growth of Human Pancreatic Cancer Cells

Keisuke Mitsuoka ^{a,b}, Yukio Kato ^a, Sosuke Miyoshi ^b, Yoshihiro Murakami ^b, Mariko Hiraiwa ^a, Yoshiyuki Kubo ^a, Shintaro Nishimura ^b, Akira Tsuji ^{a,*}

^a *Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology, Kanazawa University; Kakuma-machi, Kanazawa 920-1192 Japan*

^b *The Medical and Pharmacological Research Center Foundation, Wo 32, Inoyama-machi, Hakui 925-0613, Japan*

*Corresponding author:

Akira Tsuji, Ph.D.

Division of Pharmaceutical Sciences, Graduate School of Natural Science and Technology,
Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan

Tel: +81-76-264-5085 / Fax: +81-76-234-4010

E-mail: tsuji@kenroku.kanazawa-u.ac.jp

Abstract

Oligopeptide transporters are abundantly expressed in various types of cancer cells. We here synthesized two novel dipeptides, L-Phenylalanylsarcosine (Phe-Sar) and 4-(4-methoxyphenyl)-L-phenylalanyl sarcosine (Bip(OMe)-Sar), and examined their effect on the growth of human pancreatic cancer AsPC-1 cells, which are known to highly express oligopeptide transporter PEPT1/SLC15A1. Growth of AsPC-1 cells was inhibited by these two peptides and a typical PEPT1/SLC15A1 substrate Gly-Sar. Growth inhibition by Gly-Sar, Phe-Sar and Bip(OMe)-Sar was concentration-dependent with half-maximal inhibitory concentration of 50, 0.91 and 0.55 mM, respectively. These peptides also inhibited PEPT1-mediated [³H]Gly-Sar uptake with half-maximal inhibitory concentration of 2.6, 0.81 and 0.27 mM, respectively. Thus, the rank order of the tumor cell growth inhibition by these three peptides was the same as that of PEPT1-inhibitory activity. Growth of AsPC-1 cells was also inhibited by 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH), which is a typical inhibitor of amino acid transporter system L. The growth inhibition by BCH and Gly-Sar was additive, suggesting that these compounds act at distinct loci. Oligopeptide transporters thus appear to be a promising target for inhibition of pancreatic cancer progression. These results also proposed the idea that oligopeptide transporter is required for growth of AsPC-1 cells.

Key words: peptide transporter; dipeptides; pancreatic cancer

1. Introduction

H⁺/oligopeptide cotransporter PEPT1/SLC15A1 is functionally expressed at plasma membranes of epithelial cells in small intestine and kidney in healthy mammals. It plays an important role in the absorption of peptides and peptide-mimetic drugs such as β -lactam antibiotics (Tsuji et al., 1987a; Tsuji et al., 1987b; Tsuji et al., 1987c; Nozawa et al., 2003; Mitsuoka et al., 2007; Kato et al., 2009), anti-herpesvirus drug valacyclovir (Balimane et al., 1998; Han et al., 1998), the photosensitizer 5-aminolevulinic acid (Doring et al., 1998), and dipeptidyl derivatized L-Dopa, which is used to treat Parkinson's disease (Tsuji et al., 1990; Tamai et al., 1998; Tsuji, 1999). The other family member PEPT2 (encoded by *SLC15A2*) is predominantly expressed in epithelial cells, acting for reabsorption of dipeptides in the kidney (Rubio-Aliaga et al., 2003; Ocheltree et al., 2005; Frey et al., 2007; Shen et al., 2007) and efflux of the substrate drugs into cerebrospinal fluid in choroid plexus (Shen et al., 2003; Teuscher et al., 2004; Hu et al., 2007). PEPT1 and PEPT2 utilize an inwardly directed electrochemical proton gradient into cells to drive substrate transport.

We have previously discovered oligopeptide transport activity in a cancer cell line, human fibrosarcoma HT1080, but not in the normal fibroblast cell line IMR-90 (Nakanishi et al., 1997). Subsequent studies have shown that other cancer cell lines also express oligopeptide transporters including PEPT1 (Gonzalez et al., 1998; Nakanishi et al., 2000; Knutter et al., 2002; Inoue et al., 2005). In addition, by utilizing tumor-specific transporter expression, as compared with limited expression in normal tissues, we have recently succeeded in detection of inoculated tumors with [¹¹C]glycylsarcosine (Gly-Sar) as a positron emission tomography probe (Mitsuoka et al., 2008). Thus, [¹¹C]Gly-Sar is a candidate tumor-imaging agent, and peptide transporters are considered to be a promising target for the delivery of such imaging agents.

Interestingly, recent studies have revealed that tripeptides suppress tumor growth *in vivo* and *in vitro* (Jia et al., 2005; Lu et al., 2006; Yao et al., 2006; Shi et al., 2008). Although the molecular mechanism(s) responsible for the tumor suppression remains unknown, involvement of oligopeptide transporters seems likely, considering the broad expression profiles of PEPT1 and PEPT2 in various types of cancer cell lines (Mitsuoka et al., 2008), accepting di- and tripeptides as substrates (Biegel et al., 2006; Vig et al., 2006). It would be reasonable that di- and tripeptides are taken up as nutrients into cancer cells to support their high metabolic activity. However, little is known about the pathophysiological role of oligopeptide transporters in the tumors, and further studies are needed to clarify their roles in cancer cells.

Accordingly, we hypothesized in the present study that inhibition of oligopeptide transporters in cancer cells would suppress the growth of the cells by reducing the supply of nutrients *in vitro*. As candidate inhibitors, we used synthetic dipeptides that were expected to show affinity for PEPT1 and PEPT2, but to be resistant to degradation by peptidases. Gly-Sar, a well characterized typical substrate of PEPTs (Ocheltree et al., 2005; Hu et al., 2008), contains a *N*-methyl amide isostere and is resistant to hydrolysis. Introduction of *N*-methyl amide bioisosteres as peptide bond replacements would thus confer peptidase resistance, while having a minimal effect on the affinity for PEPTs (Andersen et al., 2006; Knutter et al., 2007). Hence we also used Phe-Sar and Bip(OMe)-Sar as candidate inhibitors. Among the various types of cancer cells expressing PEPT1 and/or PEPT2 (Mitsuoka et al., 2008), pancreatic cancer cell line AsPC-1 was selected to check growth inhibition potential of the synthetic dipeptides. Pancreatic cancer is one of the most common causes of cancer death. Due to its aggressive growth behavior with early local spread into the surrounding tissues, early metastasis, and resistance to radiation and most systemic therapies, the prognosis of

pancreatic cancer remains poor. The efficacy of postoperative therapy (chemotherapy with or without chemoradiation therapy) in the management of this disease remains controversial (Neoptolemos et al., 2001; Neoptolemos et al., 2004), and new therapeutic strategies are necessary to combat this deadly disease. In the present study, we compared inhibition potential of three synthetic dipeptides for oligopeptide transport activity and cellular growth with an aim to examine whether the oligopeptide transporter is a promising target for cancer chemotherapy.

2. Materials and Methods

2.1. Chemicals and Cells

[³H]Gly-Sar (18.5 GBq/mmol, Moravek Biochemicals Inc., Brea, CA) was obtained from Daiichi Pure Chemicals Co., Ltd. (Ibaraki, Japan). Inulin-carboxyl, [Carboxyl-¹⁴C] (161 MBq/g) was from MP Biomedicals (CostaMesa, CA). All other reagents were of the highest grade available from Sigma-Aldrich (St. Louis, MO), Wako Pure Chemicals Industries, Ltd. (Osaka, Japan), Invitrogen Corporation (Carlsbad, CA), Kanto Chemicals (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan).

2.2. Cell Culture

AsPC-1 cells (CRL-1682; American Type Culture Collection) were cultured in RPMI medium (Invitrogen) containing 10% fetal bovine serum (Hyclone), 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 1.5 g/l bicarbonate. HeLa cells stably expressing human PEPT1 (HeLa/PEPT1) or vector alone (HeLa/Mock) were previously established (Nakanishi et al., 2000) and cultured in MEM, Earle's salt (Invitrogen) containing 10% fetal bovine serum and 0.6 mg/ml geneticin.

2.3. Synthesis of Bip(OMe)-Sar and Phe-Sar

Bip(OMe)-Sar was synthesized from *N*-Boc-4-(4-methoxyphenyl)-L-phenylalanine (Sircar et al., 2002) and sarcosine benzyl ester. Phe-Sar was synthesized from *N*-Boc-L-phenylalanine and sarcosine benzyl ester. The synthetic routes are summarized in Fig. 1. Proton NMR spectra were recorded on a Varian INOVA600. δ values in parts per million relative to tetramethylsilane are given. Mass spectra were recorded with a Thermo Electron LCQ. 4-(4-Methoxyphenyl)-L-phenylalanyl sarcosine (Bip(OMe)-Sar) was synthesized as follows. To a solution of *N*-Boc-4-(4-methoxyphenyl)phenylalanine (150 mg), sarcosine benzyl ester (76 mg), and 1-hydroxybenzotriazole (HOBt) (60 mg) in DMF (3 ml) was added ethyl

N,N-dimethylaminopropyl carbodiimide monohydrochloride (WSCl) (85 mg) and the solution was stirred for 2 h. The solution was diluted with ethyl acetate, and then washed with water and brine, dried over MgSO₄, filtered, and concentrated in vacuo. The crude material was purified by column chromatography on silica gel with Et₂O/hexane (1:1) to give *N*-Boc-4-(4-methoxyphenyl)-*L*-phenylalanyl sarcosine benzyl ester (152 mg). A mixture of the obtained benzyl ester (152 mg) and 10% Pd/C (15 mg) in methanol (7.5 ml) was stirred under an H₂ atmosphere for 1 h. The mixture was filtered through Celite to remove the catalyst, and the filtrate was concentrated under reduced pressure to yield *N*-Boc-4-(4-methoxyphenyl)-*L*-phenylalanyl sarcosine (129 mg). The obtained Boc-protected dipeptide was dissolved in EtOAc (1 ml). To this solution, 4 N HCl/EtOAc (2 ml) was added and the mixture was stirred at room temperature for 1 h. The mixed solution was concentrated under reduced pressure, and the pH of the residue was adjusted to 8 with saturated aqueous NaHCO₃. The mixed solution was purified by ODS column chromatography with MeCN/H₂O (0:100 to 25:75). The eluate was concentrated in vacuo and lyophilized to give 4-(4-methoxyphenyl)-*L*-phenylalanyl sarcosine as an amorphous solid (83 mg, 60% from *N*-Boc-4-methoxyphenyl-phenylalanine): ¹H NMR (DMSO-d₆) δ 2.81 (1H, d, J = 17.4), 2.88 (1H, dd, J = 13.2, 4.2), 3.13 (1H, dd, J = 13.2, 4.2), 3.53 (1H, d, J = 17.4), 3.786 (3H, s), 3.790 (3H, s), 4.16 (1H, m), 7.00 (2H, m), 7.26 (2H, d, J = 7.8), 7.48 (2H, d, J = 7.8), 7.54-7.61 (2H, m); MS (ESI) m/z 343.1 (M+H)⁺. *L*-Phenylalanylsarcosine (Phe-Sar) was synthesized from *N*-Boc-*L*-phenylalanine and sarcosine benzyl ester using a procedure similar to that employed for the preparation of Bip(OMe)-Sar: ¹H NMR (DMSO-d₆) δ 2.60 (1H, d, J = 17.6), 2.64 (3H, s), 2.85 (1H, dd, J = 13.6, 4.8), 3.11 (1H, dd, J = 13.6, 4.8), 4.15 (1H, m), 7.09 (2H, m), 7.23-7.31 (3H, m), 8.26 (1H, s); MS (ESI) m/z 237.0 (M+H)⁺.

2.4. Cellular Uptake Study

Cells were seeded at a density of 5.0×10^4 (AsPC-1) or 2.0×10^4 (HeLa) cells per 96-well plate (Nalge Nunc) in growth media without antibiotics, and grown for 2 days. The passage numbers were in the ranges of 4 – 7 for AsPC-1 cells. Uptake of $2 \mu\text{M}$ [^3H]Gly-Sar by the cultured cells was examined at 37°C , pH 6.0 or 7.4 in Hanks' balanced salt solution. The procedures of the uptake experiments were described previously (Mitsuoka et al., 2007). In the inhibition study using unlabeled Gly-Sar or other dipeptides, the final concentration of [^3H]Gly-Sar was set to be constant ($2 \mu\text{M}$) in the presence of various concentrations of unlabeled compound. The incubation mixture was prepared by mixing the stock solution containing [^3H]Gly-Sar ($4 \mu\text{M}$) and those containing various concentration of unlabeled dipeptides at a ratio of 1 : 1 (v/v). Uptake of [^3H]Gly-Sar was measured as the radioactivity associated with the cells using a liquid scintillation counter, LSC-6100 (Aloka) and normalized by the control (without unlabeled dipeptide) value. The IC_{50} values (concentration at half-maximum inhibition of [^3H]Gly-Sar uptake) for each unlabeled dipeptide were obtained using KaleidaGraph 4.0 (Synergy Software) by fitting the data to a four-parameter logistic:

$$V_{\min} + (V_{\max} - V_{\min}) / (1 + (S/\text{IC}_{50})^n) \quad \dots \text{Equation 1}$$

where V_{\max} , V_{\min} , S and n are the maximum uptake of [^3H]Gly-Sar (without unlabeled dipeptide), the minimum uptake of [^3H]Gly-Sar (in the presence of highest concentration of dipeptide), concentration of unlabeled dipeptide and the hill constant, respectively

2.5. Membrane Potential Assay

Measurements of membrane potential in HeLa/PEPT1 or HeLa/Mock cell lines were performed as previously described (Mitsuoka et al., 2009) using a FlexStation⁹⁶ II (Nihon Molecular Devices). The EC_{50} values (concentration at half-maximum fluorescence response) were obtained using KaleidaGraph by fitting the data to a four-parameter logistic equation,

$$y = (Fl_{base} - Fl_{max}) / [1 + (x / EC_{50})^S] + Fl_{max} \quad \dots \text{Equation 2}$$

where Fl_{base} , Fl_{max} and S are baseline response, the maximum response and the slope, respectively. Because the maximum fluorescence values exhibited some day-to-day variability, all the fluorescence intensity data were normalized by the maximum value for Gly-Sar, which was routinely obtained in all experiments as a control.

2.6. Evaluation of Cell Survival (CellTiterGlo ATP production assay)

AsPC-1 cells (1×10^5 cells/100 μ l/well) were plated in 96-well plates (Packard BioScience BV) and incubated at 37 °C under 95% air/5% CO₂ for 4 h prior to drug addition to allow the cells to adhere to the bottom of the well. Cell survival was evaluated using CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega) according to the manufacturer's protocol with some modifications. The culture medium containing an appropriate concentration of test compound was replaced every day. Luminescence was measured by Wallac 1420 ARVO MX/Light (PerkinElmer Japan), and the intensity was expressed as the light counts per second (cps). The concentration required for 50% inhibition of growth (GI₅₀) values were calculated using KaleidaGraph by nonlinear data fitting to the following equation

$$y = 100 \times GI_{50} / (GI_{50} + x) \quad \dots \text{Equation 3}$$

To evaluate the effect of osmolarity on the growth of AsPC-1 cells, D-mannitol (25, 50, and 100 mM) was also included in the culture medium, and the cell survival was evaluated by the same method as described above.

2.7. Statistical Analysis

Results are presented as mean \pm S.D. The statistical significance of differences between two means was determined by Student's *t*-test, and that of differences among means of more than two groups was determined by one-way analysis of variance followed by Dunnett's post hoc

test using KaleidaGraph. Differences with a *P*-value of 0.05 or less were considered as statistically significant.

3. Results

3.1 Uptake of [³H]Gly-Sar in AsPC-1 Cells

The uptake of [³H]Gly-Sar in AsPC-1 was measured in the absence or presence of unlabeled Gly-Sar (20 mM) (Fig. 2A). Uptake of [³H]Gly-Sar increased time-dependently. The uptake was notably reduced in the presence of unlabeled Gly-Sar. The uptake was found to be linear over the 20-min incubation period, and therefore, we evaluated initial uptake at 5 min in subsequent studies. The uptake of [³H]Gly-Sar in AsPC-1 was significantly greater at acidic pH than that at neutral pH (Fig. 2B), as was the case for the transport by PEPTs.

3.2. Modulation of Membrane Potential by Gly-Sar

Modulation of membrane potential in HeLa/PEPT1 cells by addition of various concentrations of Gly-Sar was quantified. The membrane potential indicator dye yielded a time- and concentration-dependent increase of fluorescence intensity in response to Gly-Sar in HeLa/PEPT1 cells, reflecting membrane depolarization associated with proton-coupled substrate transport (Fig. 3A). There was no change in fluorescence intensity in HeLa/Mock cells after addition of Gly-Sar (data not shown). The change in fluorescence intensity from the base line was plotted against Gly-Sar concentration; the plots indicated saturable kinetics in HeLa/PEPT1 cells, but not in HeLa/Mock cells (Fig. 3B).

3.3. Differences in Modulation of Membrane Potential by Synthetic Dipeptides

Two additional synthetic dipeptides were also tested for the ability to activate PEPT1. In Fig. 4A, PEPT1 activation is represented by the dose-dependent increase in fluorescence intensity caused by Gly-Sar and Phe-Sar; however, no activation was observed following the addition of Bip(OMe)-Sar, showing that Bip(OMe)-Sar was not transported by PEPT1. The EC₅₀ and *Fl*_{max} values obtained by nonlinear regression analysis are summarized in Table 1, and indicate that Phe-Sar has higher affinity than Gly-Sar for PEPT1.

3.4. Effect of Synthetic Dipeptides on [³H]Gly-Sar Uptake

The inhibitory effects of Gly-Sar, Phe-Sar and Bip(OMe)-Sar on PEPT1-mediated transport were studied in HeLa/PEPT1 cells using [³H]Gly-Sar as a typical substrate. In contrast to membrane potential studies, all the tested dipeptides inhibited the [³H]Gly-Sar uptake in HeLa/PEPT1 (Fig. 4B). The rank order of inhibitory potential was Bip(OMe)-Sar > Phe-Sar > Gly-Sar (Fig. 4B and Table 1).

3.5 Inhibitory Effect of Synthetic Dipeptides on the Growth of AsPC-1 Cells

To examine the cytotoxic and/or cytostatic effect of synthetic dipeptides, AsPC cells were cultured in the presence of each peptide, and the number of cells was quantified in terms of luminescence due to cellular ATP. Bip(OMe)-Sar and Gly-Sar suppressed growth of AsPC-1, and the inhibition occurred at lower concentration (1 mM) in the case of Bip(OMe)-Sar than Gly-Sar (100 mM) (Fig. 5). At the day 3, only 8.2% and 25.1% growth of the cells was achieved in the presence of Gly-Sar and Bip(OMe)-Sar, respectively. A synthetic leucine analogue, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH) induced depletion of ATP, exhibiting a cytotoxic effect as reported previously (Nawashiro et al., 2006). When AsPC-1 cells were cultured in medium containing both Gly-Sar and BCH, growth of the cells was further suppressed as compared with BCH alone (Table 2). Thus, the inhibitory effects of Gly-Sar and BCH appeared to be additive (Table 2).

4. Discussion

Oligopeptide transporters PEPT1 and PEPT2 are expressed in various types of human cancer cell lines, including pancreatic carcinoma (Gonzalez et al., 1998; Nakanishi et al., 2000; Knutter et al., 2002; Inoue et al., 2005; Mitsuoka et al., 2008), and greater amount of peptide was reported to be accumulated in cancer cells compared with normal cells (Nakanishi et al., 1997); this would be consistent with function for nutrient uptake. This implies that a strategy of inhibition of PEPTs may result in decreased cellular growth of tumors. In the present study, we examined whether inhibition of PEPTs can suppress the *in vitro* growth of cancer cells using human pancreatic cancer cell line AsPC-1, which highly expresses PEPT1 (Gonzalez et al., 1998). These cells showed efficient uptake of a typical substrate, Gly-Sar, in a pH-dependent manner (Fig. 2). We found that the three dipeptides examined here inhibited both PEPT1-mediated transport (Fig. 4B) and growth of AsPC-1 cells in a concentration-dependent manner (Fig. 5, Table 1). Thus, the present findings suggest that a strategy of inhibiting cancer cell growth by using inhibitors of PEPTs might be effective.

Membrane potential assay revealed that Phe-Sar is a substrate of PEPT1 (Fig. 3). This assay is based on measurement of membrane depolarization resulting from the cotransport of protons and PEPT1 substrate (Faria et al., 2004; Vig et al., 2006). Plots of concentration dependency indicated that Phe-Sar has a lower EC₅₀ value, and therefore, a higher affinity for PEPT1 than Gly-Sar (Table 1). On the other hand, Bip(OMe)-Sar inhibited [³H]Gly-Sar uptake (Fig. 4B) without modulating the membrane potential (Fig. 4A). Thus, Bip(OMe)-Sar is a non-transportable inhibitor of PEPT1. Bip(OMe)-Sar exhibited the lowest IC₅₀ value, and is therefore the most potent inhibitor among the three dipeptides (Table 1). Because AsPC-1 cells functionally express H⁺-dependent oligopeptide transport activity (Fig. 2), we also

evaluated the effects of this compound on cell growth. AsPC-1 cells exhibited remarkable growth under normal culture conditions, whereas their growth was almost completely blocked in the presence of Bip(OMe)-Sar at 1 mM. The cell growth was also inhibited in the presence of Gly-Sar, but the growth inhibition by 1 mM Bip(OMe)-Sar was similar to that by 100 mM Gly-Sar (Fig. 5). This is consistent with the much higher affinity (lower IC_{50}) of Bip(OMe)-Sar than Gly-Sar for PEPT1 (Fig. 4 and Table 1). We confirmed that this growth inhibitory effect of the Gly-Sar on AsPC-1 cells was probably not due to high osmolarity of the culture medium added with Gly-Sar since addition of the equivalent molarity of D-mannitol showed no remarkable effect on the growth of the cells (data not shown). Because the rank order of GI_{50} values for AsPC-1 cells was Bip(OMe)-Sar > Phe-Sar > Gly-Sar, and the same rank order was also obtained for inhibition of PEPT1-mediated transport (IC_{50}), it appears that inhibitory potential towards PEPT1 is important for the growth inhibition of AsPC-1 cells. However, the present findings cannot rule out the possibility that direct toxic effects of these dipeptides are also relevant to the growth inhibition of AsPC-1 cells. Therefore, further experiments are necessary to clarify the biological roles of PEPT1 in the growth of AsPC-1 cells. In addition, the GI_{50} value for Gly-Sar was about 50-fold higher than that for Phe-Sar, whereas the IC_{50} values for PEPT1 inhibition exhibited only three-times difference between Gly-Sar and Phe-Sar (Table 1), suggesting that the growth inhibition of AsPC-1 cells cannot be simply explained by the inhibition of PEPT1-mediated transport alone.

In contrast to the dipeptides, BCH decreased the ATP content in AsPC-1 cells (Fig. 5), indicating the induction of cell death. BCH is a potent inhibitor for the system L amino acid transporter, which could be another molecular target for suppression of tumor growth (Nawashiro et al., 2006; Kim et al., 2008). The system L functions as an uptake transporter

for nutrients, and inhibition of the transporter is expected to hinder cell growth. In the present study, cell growth was more potently suppressed by concomitant use of Gly-Sar and BCH, as compared with the use of Gly-Sar or BCH alone (Table 2). Such an additive effect may suggest that the compounds act at distinct loci, i.e., inhibition of oligopeptide transporter and system L may lead to distinct intracellular events. This would indicate that the combination of inhibition of the two transporters might be an effective approach for cancer chemotherapy.

Cancer cell lines such as moderately differentiated pancreatic adenocarcinoma AsPC-1 or poorly differentiated gastric cancer MKN45 can survive for longer than normal cells in the absence of essential nutrients such as amino acids and glucose (Izuishi et al., 2000). These two cell lines highly express oligopeptide transporter PEPT1 (Mitsuoka et al., 2008), which may act as an uptake mechanism for oligopeptides as alternative nutrients. In addition, when MKN45 cells were treated with a chemotherapeutic agent, 5-FU, gene expression of PEPT1 and oligopeptide transport activity increased concomitantly with a decrease in the expression of facilitative glucose transporter and glucose uptake (Inoue et al., 2005). These observations suggest that the resistance of certain tumor cells to depletion of glucose or amino acids, or to chemotherapy, might be associated with enhanced metabolism of oligopeptides taken up via oligopeptide transporters. Pancreatic cancer is generally classed as a tumor that is highly resistant to chemotherapy. Nevertheless, our results indicate that inhibitors of oligopeptide transporter are promising candidates to suppress the growth of pancreatic cancer cells. In addition, it is likely that the inhibition of oligopeptide transporters *in vivo* would cause minimal side effects, because PEPT1 and PEPT2-deficient mice were found to be viable and fertile, and grew to normal size and weight without any obvious abnormalities (Shen et al., 2003; Shen et al., 2007; Hu et al., 2008). Huang et al. (Huang et al., 2004) analyzed correlations between gene expression of transporters and chemosensitivity in 60 cancer cell

lines established by the National Cancer Institute (NCI60) for drug screening. A positive correlation was observed between *SLC15A1* expression and potency of some anticancer agents, including fluorodopan and teroxirone. This further supports the idea that inhibition and/or modulation of PEPT1 might be a promising approach for cancer chemotherapy. However, the growth inhibition of AsPC-1 cells by Bip(OMe)-Sar and Phe-Sar was observed at mM range in the present study (Table 1). In light of the concentrations at which growth inhibition effects are seen, it appears that much more potent inhibitors would be needed to apply the present findings *in vitro* to the clinical application *in vivo*.

In conclusion, our results indicate that oligopeptide transporters are required to maintain AsPC-1 cell growth, and thus may be a suitable target for inhibition of human pancreatic cancer progression.

Acknowledgements

This study was sponsored by Ishikawa prefectural government, Japan, and also supported in part by a Grant-in-Aid for Scientific Research provided by the Ministry of Education, Science and Culture of Japan, and in part by the Sagawa Foundation for Promotion of Cancer Research.

References

Andersen, R., Nielsen, C.U., Begtrup, M., Jorgensen, F.S., Brodin, B., Frokjaer, S. Steffansen, B., 2006. In vitro evaluation of N-methyl amide tripeptidomimetics as substrates for the human intestinal di-/tri-peptide transporter hPEPT1. *Eur. J. Pharm. Sci.* 28, 325-335.

- Balimane, P.V., Tamai, I., Guo, A., Nakanishi, T., Kitada, H., Leibach, F.H., Tsuji, A. Sinko, P.J., 1998. Direct evidence for peptide transporter (PepT1)-mediated uptake of a nonpeptide prodrug, valacyclovir. *Biochem. Biophys. Res. Commun.* 250, 246-251.
- Biegel, A., Gebauer, S., Brandsch, M., Neubert, K. Thondorf, I., 2006. Structural requirements for the substrates of the H⁺/peptide cotransporter PEPT2 determined by three-dimensional quantitative structure-activity relationship analysis. *J. Med. Chem.* 49, 4286-4296.
- Doring, F., Walter, J., Will, J., Focking, M., Boll, M., Amasheh, S., Clauss, W. Daniel, H., 1998. Delta-aminolevulinic acid transport by intestinal and renal peptide transporters and its physiological and clinical implications. *J. Clin. Invest.* 101, 2761-2767.
- Faria, T.N., Timoszyk, J.K., Stouch, T.R., Vig, B.S., Landowski, C.P., Amidon, G.L., Weaver, C.D., Wall, D.A. Smith, R.L., 2004. A novel high-throughput pepT1 transporter assay differentiates between substrates and antagonists. *Mol. Pharm.* 1, 67-76.
- Frey, I.M., Rubio-Aliaga, I., Siewert, A., Sailer, D., Drobyshev, A., Beckers, J., de Angelis, M.H., Aubert, J., Bar Hen, A., Fiehn, O., Eichinger, H.M. Daniel, H., 2007. Profiling at mRNA, protein, and metabolite levels reveals alterations in renal amino acid handling and glutathione metabolism in kidney tissue of *Pept2^{-/-}* mice. *Physiol. Genomics.* 28, 301-310.
- Gonzalez, D.E., Covitz, K.M., Sadee, W. Mrsny, R.J., 1998. An oligopeptide transporter is expressed at high levels in the pancreatic carcinoma cell lines AsPc-1 and Capan-2. *Cancer Res.* 58, 519-525.
- Han, H., de Vrueh, R.L., Rhie, J.K., Covitz, K.M., Smith, P.L., Lee, C.P., Oh, D.M., Sadee, W. Amidon, G.L., 1998. 5'-Amino acid esters of antiviral nucleosides, acyclovir, and

- AZT are absorbed by the intestinal PEPT1 peptide transporter. *Pharm. Res.* 15, 1154-1159.
- Hu, Y., Shen, H., Keep, R.F. Smith, D.E., 2007. Peptide transporter 2 (PEPT2) expression in brain protects against 5-aminolevulinic acid neurotoxicity. *J. Neurochem.* 103, 2058-2065.
- Hu, Y., Smith, D.E., Ma, K., Jappara, D., Thomas, W. Hillgren, K.M., 2008. Targeted Disruption of Peptide Transporter Pept1 Gene in Mice Significantly Reduces Dipeptide Absorption in Intestine. *Mol. Pharm.* 5, 1122-1130.
- Huang, Y., Anderle, P., Bussey, K.J., Barbacioru, C., Shankavaram, U., Dai, Z., Reinhold, W.C., Papp, A., Weinstein, J.N. Sadee, W., 2004. Membrane transporters and channels: role of the transportome in cancer chemosensitivity and chemoresistance. *Cancer Res.* 64, 4294-4301.
- Inoue, M., Terada, T., Okuda, M. Inui, K., 2005. Regulation of human peptide transporter 1 (PEPT1) in gastric cancer cells by anticancer drugs. *Cancer Lett.* 230, 72-80.
- Izuishi, K., Kato, K., Ogura, T., Kinoshita, T. Esumi, H., 2000. Remarkable tolerance of tumor cells to nutrient deprivation: possible new biochemical target for cancer therapy. *Cancer Res.* 60, 6201-6207.
- Jia, J., Lu, R., Qiu, S., Li, H., Che, X., Zhao, P., Jin, M., Yang, H., Lin, G. Yao, Z., 2005. Preliminary investigation of the inhibitory effects of the tyroservaltide (YSV) tripeptide on human hepatocarcinoma BEL-7402. *Cancer Biol. Ther.* 4, 993-997.
- Kato, Y., Sugiura, T., Nakadera, Y., Sugiura, M., Kubo, Y., Sato, T., Harada, A. Tsuji, A., 2009. Investigation of the role of oligopeptide transporter PEPT1 and sodium/glucose cotransporter SGLT1 in intestinal absorption of their substrates using small GTP-binding protein Rab8-null mice. *Drug Metab. Dispos.* 37, 602-607.

- Kim, C.S., Cho, S.H., Chun, H.S., Lee, S.Y., Endou, H., Kanai, Y. Kim do, K., 2008. BCH, an inhibitor of system L amino acid transporters, induces apoptosis in cancer cells. *Biol. Pharm. Bull.* 31, 1096-1100.
- Knutter, I., Hartrodt, B., Toth, G., Keresztes, A., Kottra, G., Mrestani-Klaus, C., Born, I., Daniel, H., Neubert, K. Brandsch, M., 2007. Synthesis and characterization of a new and radiolabeled high-affinity substrate for H⁺/peptide cotransporters. *FEBS J.* 274, 5905-5914.
- Knutter, I., Rubio-Aliaga, I., Boll, M., Hause, G., Daniel, H., Neubert, K. Brandsch, M., 2002. H⁺-peptide cotransport in the human bile duct epithelium cell line SK-ChA-1. *Am. J. Physiol. Gastrointest. Liver Physiol.* 283, G222-229.
- Lu, R., Jia, J., Bao, L., Fu, Z., Li, G., Wang, S., Wang, Z., Jin, M., Gao, W. Yao, Z., 2006. Experimental study of the inhibition of human hepatocarcinoma Bel7402 cells by the tripeptide tyrosyleutide(YSL). *Cancer Chemother. Pharmacol.* 57, 248-256.
- Mitsuoka, K., Kato, Y., Kubo, Y. Tsuji, A., 2007. Functional expression of stereoselective metabolism of cephalexin by exogenous transfection of oligopeptide transporter PEPT1. *Drug Metab. Dispos.* 35, 356-362.
- Mitsuoka, K., Miyoshi, S., Kato, Y., Murakami, Y., Utsumi, R., Kubo, Y., Noda, A., Nakamura, Y., Nishimura, S. Tsuji, A., 2008. Cancer detection using a PET tracer, ¹¹C-glycylsarcosine, targeted to H⁺/peptide transporter. *J. Nucl. Med.* 49, 615-622.
- Mitsuoka, K., Tamai, I., Morohashi, Y., Kubo, Y., Saitoh, R., Tsuji, A. Kato, Y., 2009. Direct evidence for efficient transport and minimal metabolism of L-cephalexin by oligopeptide transporter 1 in budded baculovirus fraction. *Biol. Pharm. Bull.* 32, 1459-1461.

- Nakanishi, T., Tamai, I., Sai, Y., Sasaki, T. Tsuji, A., 1997. Carrier-mediated transport of oligopeptides in the human fibrosarcoma cell line HT1080. *Cancer Res.* 57, 4118-4122.
- Nakanishi, T., Tamai, I., Takaki, A. Tsuji, A., 2000. Cancer cell-targeted drug delivery utilizing oligopeptide transport activity. *Int. J. Cancer* 88, 274-280.
- Nawashiro, H., Otani, N., Shinomiya, N., Fukui, S., Ooigawa, H., Shima, K., Matsuo, H., Kanai, Y. Endou, H., 2006. L-type amino acid transporter 1 as a potential molecular target in human astrocytic tumors. *Int. J. Cancer* 119, 484-492.
- Neoptolemos, J.P., Dunn, J.A., Stocken, D.D., Almond, J., Link, K., Beger, H., Bassi, C., Falconi, M., Pederzoli, P., Dervenis, C., Fernandez-Cruz, L., Lacaine, F., Pap, A., Spooner, D., Kerr, D.J., Friess, H. Buchler, M.W., 2001. Adjuvant chemoradiotherapy and chemotherapy in resectable pancreatic cancer: a randomised controlled trial. *Lancet* 358, 1576-1585.
- Neoptolemos, J.P., Stocken, D.D., Friess, H., Bassi, C., Dunn, J.A., Hickey, H., Beger, H., Fernandez-Cruz, L., Dervenis, C., Lacaine, F., Falconi, M., Pederzoli, P., Pap, A., Spooner, D., Kerr, D.J. Buchler, M.W., 2004. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N. Engl. J. Med.* 350, 1200-1210.
- Nozawa, T., Toyobuku, H., Kobayashi, D., Kuruma, K., Tsuji, A. Tamai, I., 2003. Enhanced intestinal absorption of drugs by activation of peptide transporter PEPT1 using proton-releasing polymer. *J. Pharm. Sci.* 92, 2208-2216.
- Ocheltree, S.M., Shen, H., Hu, Y., Keep, R.F. Smith, D.E., 2005. Role and relevance of peptide transporter 2 (PEPT2) in the kidney and choroid plexus: in vivo studies with

- glycylsarcosine in wild-type and PEPT2 knockout mice. *J. Pharmacol. Exp. Ther.* 315, 240-247.
- Rubio-Aliaga, I., Frey, I., Boll, M., Groneberg, D.A., Eichinger, H.M., Balling, R. Daniel, H., 2003. Targeted disruption of the peptide transporter *Pept2* gene in mice defines its physiological role in the kidney. *Mol. Cell. Biol.* 23, 3247-3252.
- Shen, H., Ocheltree, S.M., Hu, Y., Keep, R.F. Smith, D.E., 2007. Impact of genetic knockout of PEPT2 on cefadroxil pharmacokinetics, renal tubular reabsorption, and brain penetration in mice. *Drug Metab. Dispos.* 35, 1209-1216.
- Shen, H., Smith, D.E., Keep, R.F., Xiang, J. Brosius, F.C., 3rd, 2003. Targeted disruption of the PEPT2 gene markedly reduces dipeptide uptake in choroid plexus. *J. Biol. Chem.* 278, 4786-4791.
- Shi, L.X., Ma, R., Lu, R., Xu, Q., Zhu, Z.F., Wang, L., Zhou, C.L., Li, X.L., Zhang, H.L. Yao, Z., 2008. Reversal effect of tyroservatide (YSV) tripeptide on multi-drug resistance in resistant human hepatocellular carcinoma cell line BEL-7402/5-FU. *Cancer Lett.* 269, 101-110.
- Sircar, I., Gudmundsson, K.S., Martin, R., Liang, J., Nomura, S., Jayakumar, H., Teegarden, B.R., Nowlin, D.M., Cardarelli, P.M., Mah, J.R., Connell, S., Griffith, R.C. Lazarides, E., 2002. Synthesis and SAR of N-benzoyl-L-biphenylalanine derivatives: discovery of TR-14035, a dual $\alpha(4)\beta(7)/\alpha(4)\beta(1)$ integrin antagonist. *Bioorg. Med. Chem.* 10, 2051-2066.
- Tamai, I., Nakanishi, T., Nakahara, H., Sai, Y., Ganapathy, V., Leibach, F.H. Tsuji, A., 1998. Improvement of L-dopa absorption by dipeptidyl derivation, utilizing peptide transporter *PepT1*. *J. Pharm. Sci.* 87, 1542-1546.

- Teuscher, N.S., Shen, H., Shu, C., Xiang, J., Keep, R.F. Smith, D.E., 2004. Carnosine uptake in rat choroid plexus primary cell cultures and choroid plexus whole tissue from PEPT2 null mice. *J. Neurochem.* 89, 375-382.
- Tsuji, A., 1999. Tissue selective drug delivery utilizing carrier-mediated transport systems. *J. Control. Release* 62, 239-244.
- Tsuji, A., Hirooka, H., Terasaki, T., Tamai, I. Nakashima, E., 1987a. Saturable uptake of cefixime, a new oral cephalosporin without an alpha-amino group, by the rat intestine. *J. Pharm. Pharmacol.* 39, 272-277.
- Tsuji, A., Tamai, I., Hirooka, H. Terasaki, T., 1987b. Beta-lactam antibiotics and transport via the dipeptide carrier system across the intestinal brush-border membrane. *Biochem. Pharmacol.* 36, 565-567.
- Tsuji, A., Tamai, I., Nakanishi, M. Amidon, G.L., 1990. Mechanism of absorption of the dipeptide alpha-methyldopa-phe in intestinal brush-border membrane vesicles. *Pharm. Res.* 7, 308-309.
- Tsuji, A., Terasaki, T., Tamai, I. Hirooka, H., 1987c. H⁺ gradient-dependent and carrier-mediated transport of cefixime, a new cephalosporin antibiotic, across brush-border membrane vesicles from rat small intestine. *J. Pharmacol. Exp. Ther.* 241, 594-601.
- Vig, B.S., Stouch, T.R., Timoszyk, J.K., Quan, Y., Wall, D.A., Smith, R.L. Faria, T.N., 2006. Human PEPT1 pharmacophore distinguishes between dipeptide transport and binding. *J. Med. Chem.* 49, 3636-3644.
- Yao, Z., Lu, R., Jia, J., Zhao, P., Yang, J., Zheng, M., Lu, J., Jin, M., Yang, H. Gao, W., 2006. The effect of tripeptide tyrosyleutide (YSL) on animal models of hepatocarcinoma. *Peptides* 27, 1167-1172.

Table 1

Comparison of inhibition of PEPT1-mediated Gly-Sar uptake, membrane potential modulation, and growth inhibition of AsPC-1 cells by synthetic dipeptides

Dipeptides	IC ₅₀ (mM) ^{a)}	EC ₅₀ (mM) ^{b)}	GI ₅₀ (mM) ^{c)}
Gly-Sar	2.62 ± 1.99	0.608 ± 0.203	49.9 ± 19.3
Phe-Sar	0.807 ± 0.174	0.263 ± 0.131	0.908 ± 0.758
Bip(OMe)-Sar	0.274 ± 0.047	N/A	0.545 ± 0.163

^{a)} Half-maximal inhibitory concentration of dipeptides for PEPT1-mediated [³H]Gly-Sar uptake determined based on Equation 1. Mean ± calculated S.D. (*n* = 3)

^{b)} Half-maximal effective concentration of dipeptides for membrane potential change determined based on Equation 2. Mean ± S.D. (*n* = 9 and 4 for Gly-Sar and Phe-Sar, respectively). N/A, No activity

^{c)} Half-maximal inhibitory concentration of dipeptides for growth inhibition of AsPC-1 cells on day 3 determined based on Equation 3. Mean ± calculated S.D. (*n* = 3)

Table 2

Effect of the combination of inhibitors of oligopeptide and amino acid transporters on the growth of AsPC-1 cells ^{a)}

Inhibitors (mM)	Growth (% of Control) ^{b)}	<i>P</i> Value (vs. Control)	<i>P</i> Value (vs. BCH)
Control	100 ± 20	–	–
Gly-Sar (25)	82.1 ± 22.1	0.179	0.020
BCH (25)	43.4 ± 9.6	< 0.001	–
Gly-Sar (25) + BCH (25)	28.8 ± 9.3	< 0.0001	0.043

^{a)} AsPC-1 cells (1×10^4 cells/well) were cultured for three days on a 96-well plate in the presence or absence of inhibitor(s) as indicated.

^{b)} The cell survival was quantified using CellTiter-Glo luminescent cell viability assay and normalized by the value in the absence of the inhibitor. Statistical significance was determined by ANOVA followed by Dunnett's post hoc test. Mean ± S.D., Control ($n = 9$); Gly-Sar ($n = 4$); BCH ($n = 4$); Gly-Sar + BCH ($n = 6$)

Legends for Figures

Figure 1

Synthetic route for Bip(OMe)-Sar and Phe-Sar.

Figure 2

Time profile of [³H]Gly-Sar uptake in AsPC-1 (A) and effect of extracellular pH on the [³H]Gly-Sar uptake (B).

(A) Cellular uptake of [³H]Gly-Sar (2 μM, 37 kBq/ml) in AsPC-1 cells was measured for 20 min at pH 6.0 in the presence (open symbols) or absence (closed symbols) of 20 mM unlabeled Gly-Sar. (B) Cellular uptake of [³H]Gly-Sar in AsPC-1 cells were measured after 5 min incubation at pH 6.0 or 7.4 in the presence (open columns) or absence (closed column) of 20 mM unlabeled Gly-Sar. Uptake of [³H]Gly-Sar was normalized by the medium concentration to give the distribution volume with dimensions of microliters per milligram of protein. An asterisk (*) indicates a significant difference from the control by Student's *t*-test ($P < 0.05$). Mean ± S.D. ($n = 4$).

Figure 3

Modulation of membrane potential by Gly-Sar in HeLa/PEPT1 cells.

Membrane potential assay was performed using a fluorometric imaging plate reader, FlexStation II⁹⁶, at pH 6.0. Gly-Sar (0 – 5000 μM) was added to HeLa/PEPT1 cells preloaded with membrane potential assay dye, and the fluorescence intensity was measured in real-time. In panel A, fluorescence intensity was plotted against time. In panel B, the data obtained at

180 sec in HeLa/PEPT1 (closed symbols) and mock cells (open symbols) were plotted against Gly-Sar concentration in the medium. Mean \pm S.D. ($n = 3$).

Figure 4

Effect of synthetic dipeptides on membrane potential (A) and [³H]Gly-Sar uptake (B) in HeLa/PEPT1 cells

(A) Modulation of membrane potential by Gly-Sar (closed circles), Phe-Sar (open diamonds), and Bip(OMe)-Sar (open triangles) was quantified using a fluorometric imaging plate reader for 180 sec at pH 6.0. Peak fluorescence intensity corrected for basal fluorescence intensity was plotted against the compound concentration. All the data were normalized by the maximum value for Gly-Sar. Mean \pm S.D. ($n = 4$). (B) Inhibitory effect of Gly-Sar (closed circles), Phe-Sar (open diamonds), and Bip(OMe)-Sar (open triangles) on the cellular uptake of [³H]Gly-Sar (2 μ M, 37 kBq/ml) was measured for 5 min at pH 6.0. The PEPT1-mediated uptake was obtained by subtraction of the uptake by mock cells from that by HeLa/PEPT1 cells and expressed as percentage of control value in the absence of inhibitors. Mean \pm S.D. ($n = 4$).

Figure 5

Effect of synthetic dipeptides on the growth of AsPC-1 cells

AsPC-1 cells (1×10^4 cells/well) were cultured on a 96-well plate in the presence of 100 mM Gly-Sar (closed circles), 1 mM Bip(OMe)-Sar (open triangles) or in their absence (open circles). After the designated days of culture, cell survival was evaluated using CellTiter-Glo luminescent cell viability assay and expressed as luminescence detected with ARVO MX/Light Wallac 1420 Multilabel Counter. The effect of 100 mM BCH, an inhibitor of

amino acid transporters, was also evaluated (open squares). Statistical significance was determined by ANOVA followed by Dunnett's post hoc test. *; $P < 0.05$ vs. control (without compound). Mean \pm S.D. ($n = 4 - 8$)

Figure 2

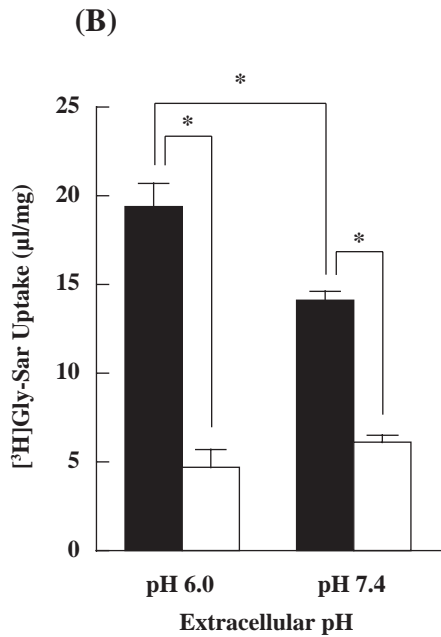
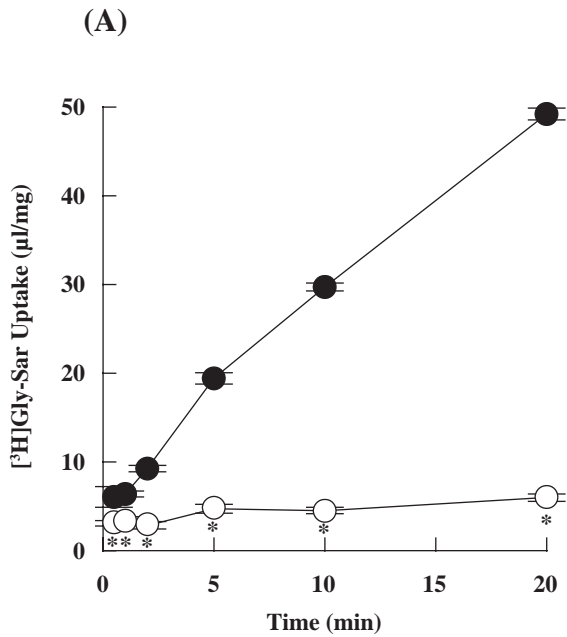


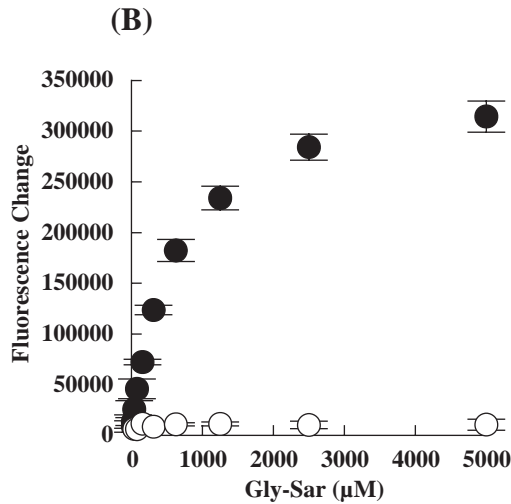
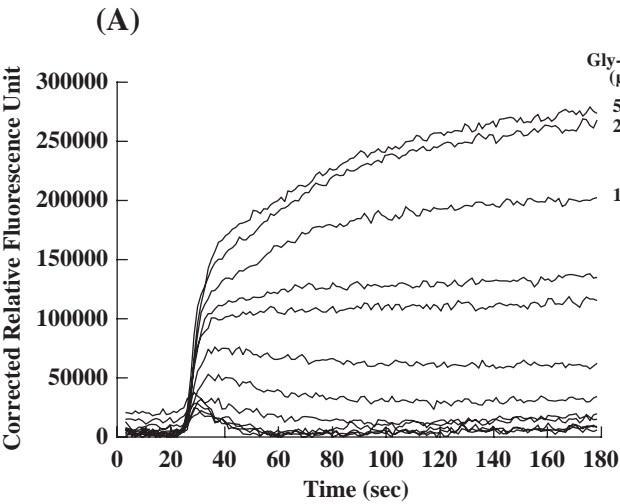
Figure 3

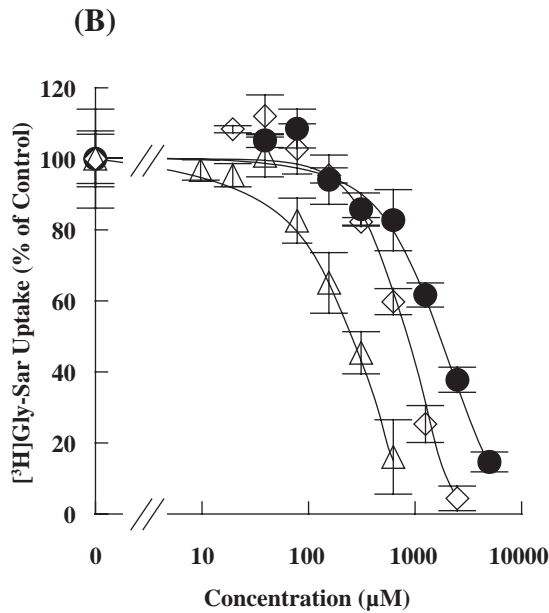
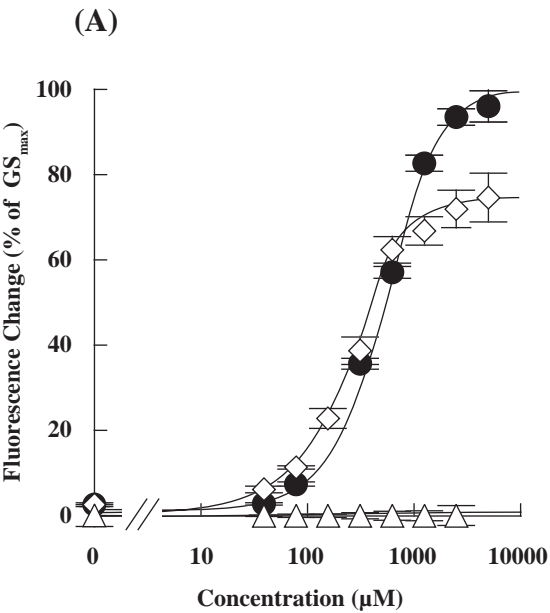
Figure 4

Figure 5

