Identification of transporters associated with etoposide sensitivity of stomach cancer cell lines and methotrexate sensitivity of breast cancer cell lines by quantitative targeted absolute proteomics

Wataru Obuchi, Sumio Ohtsuki, Yasuo Uchida, Ken Ohmine, Takao Yamori, Tetsuya Terasaki

Affiliations

Division of Membrane Transport and Drug Targeting, Department of Biochemical Pharmacology and Therapeutics, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan (W.O., S.O., Y.U., K.O., T.T.); Department of Pharmaceutical Microbiology, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan (S.O.); Division of Molecular Pharmacology, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Koto-ku, Tokyo, Japan (T.Y.)
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Key transporters for etoposide and methotrexate sensitivity

Address correspondence to: Prof. Sumio Ohtsuki, Department of Pharmaceutical Microbiology, Faculty of Life Sciences, Kumamoto University, 5-1 Oe-honmachi, Chuo-ku, Kumamoto 862-0973, Japan; Phone: +81-96-371-4323; Fax: +81-96-371-4329; Email: sohtsuki@kumamoto-u.ac.jp

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Nonstandard abbreviations

2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; 4F2hc, 4F2 heavy chain; ABC, ATP-binding cassette; ATA1, amino acid transporter A1; BCRP, breast cancer resistance protein; C/M, cell-to-medium; DHFR, dihydrofolate reductase; ENT, equilibrative nucleoside transporter; FBS, fetal bovine serum; FPGS, folylpolyglutamate synthase; GGH, gamma-glutamyl hydrolase; GI50, 50% growth-inhibitory concentration; GLUT, glucose transporter; HPLC, high-performance liquid chromatography; IC50, 50% inhibitory concentration; LAT, L-type amino acid
transporter; LC-MS/MS, liquid chromatography-linked tandem mass spectrometry; LTC4, leukotriene C4; MCT, monocarboxylate transporter; MDR1, multidrug resistance protein 1; MRM, multiple reaction monitoring; MRP, multidrug resistance-associated protein; MTX, methotrexate; MTX-Glun, methotrexate polyglutamate; OATP, organic anion-transporting polypeptide; PCFT, proton-coupled folate transporter; QTAP, quantitative targeted absolute proteomics; RFC, reduced folate carrier; RLIP76, 76 kDa Ral-interacting protein; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SLC, solute carrier; SRM, selected reaction monitoring; U.L.Q., under the limit of quantification; UPLC, ultra-performance liquid chromatography; γ-gtp, gamma-glutamyl transpeptidase
Abstract

Membrane transporter proteins may influence the sensitivity of cancer cells to anti-cancer drugs that can be recognized as substrates. The purpose of this study was to identify proteins that play a key role in the drug sensitivity of stomach and breast cancer cell lines by measuring the absolute protein expression levels of multiple transporters and other membrane proteins, and examining their correlation to drug sensitivity. Absolute protein expression levels of 90 membrane proteins were examined by quantitative targeted absolute proteomics using liquid chromatography-linked tandem mass spectrometry. Among them, 11 and 14 membrane proteins, including transporters, were present in quantifiable amounts in membrane fraction of stomach cancer and breast cancer cell lines, respectively. In stomach cancer cell lines, the protein expression level of multidrug resistance-associated protein 1 (MRP1) was inversely correlated with etoposide sensitivity. MK571, a MRPs inhibitor, increased both the cell-to-medium ratio of etoposide and the etoposide sensitivity of MRP1-expressing stomach cancer cell lines. In breast cancer cell lines, the protein expression level of reduced folate carrier 1 (RFC1) was directly correlated with methotrexate (MTX) sensitivity. Initial uptake rate and steady-state cell-to-medium ratio of \(^{3}\)HMTX were correlated with both RFC1 expression level and MTX sensitivity. These results suggest that MRP1 modulates the etoposide sensitivity of stomach cancer cell lines and RFC1 modulates the MTX sensitivity of breast cancer cell lines. Our results indicate that absolute quantification of multiple membrane proteins could be a useful strategy for identification of candidate proteins involved in drug sensitivity.
**Introduction**

Membrane transporters play an important role in determining the sensitivity of cells to various anti-cancer drugs by mediating transport of drugs across the plasma membrane. ATP-binding cassette (ABC) transporter proteins, including multidrug resistance-associated protein 1 (MDR1), the multidrug resistance-associated protein (MRP) family, and breast cancer resistance protein (BCRP), are involved in multi-drug resistance by mediating efflux of anti-cancer drugs, based on their broad specificity of substrate recognition (Szakacs et al., 2006). Solute carrier (SLC) transporter proteins also play roles in anti-cancer drug sensitivity. Equilibrative nucleoside transporter 1 (ENT1) mRNA was found to be down-regulated in cell lines resistant to cytarabine, a nucleoside analog anti-cancer drug (Takagaki et al., 2004). Down-regulation of L-type amino acid transporter 1 (LAT1) mRNA was also reported to be involved in resistance to melphalan (Kuhne et al., 2009). These findings suggest that identification of key transporter(s) for each anti-cancer drug is important to understand the molecular mechanisms of anti-cancer drug resistance and to allow selection of effective drugs for cancer chemotherapy.

The membrane transporter family consists of 48 ABC transporters and 378 SLC transporters. As drug transport often involves multiple transporters in cancer cells, it is important to identify the transporters that determine drug sensitivity. An omics strategy would be one way to identify the responsible transporters, for example, using genomics to compare mRNA expression of transporters. Genome-wide association studies by O’Brien et al. demonstrated that overexpression of ABCC3 mRNA in breast cancer cell lines is associated with reduced sensitivity to paclitaxel (O’Brien et al., 2008). However, we recently showed that protein expression of 12 transporters in plasma membrane...
fraction showed little or no correlation to mRNA expression in human livers from 17 donors (Ohtsuki et al., 2012). This clearly indicated the necessity to focus on protein expression for identifying responsible transporters.

However, it is difficult to quantitatively evaluate protein expression of transporters by means of standard proteomics techniques due to their low expression levels and problems with 2D-PAGE analysis of membrane proteins. Recently, we have reported simultaneous quantification of multiple proteins by selected reaction monitoring/multiple reaction monitoring (SRM/MRM) on the basis of liquid chromatography-linked tandem mass spectrometry (LC-MS/MS) for quantitative targeted absolute proteomics (QTAP) (Kamiie et al., 2008). In contrast to standard proteomics, which seeks to identify proteins as comprehensively as possible, QTAP enabled us to quantify the absolute expression levels of target proteins, including transporters, with increased sensitivity. We have already employed this method to quantify 22 transporters in plasma membrane fraction of human liver and 16 transporters in whole cell lysate of human brain microvessels (Ohtsuki et al., 2012; Uchida et al., 2011). Thus, this method should be suitable to compare protein expression levels of transporters in plasma membrane fraction of cancer cells in order to identify key transporters associated with drug sensitivity.

In the present study, we used QTAP to analyze protein expression of transporters in stomach and breast cancer cell lines in panels of human cancer cell lines (JFCR-39 and JFCR-45, Nakatsu et al., 2005; Yamori, 2003), since large differences of drug sensitivity have been reported in these two groups of cancer cell lines. The transporter(s) associated with drug sensitivity were identified by comparing protein expression and drug sensitivity data. Furthermore, the roles of the identified transporters were
confirmed by means of cellular uptake and viability studies.
Materials and Methods

Reagents

[3',5',7-3H]Methotrexate disodium salt ([3H]MTX, 27.7 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Ammonium salts of 4-amino-10-methylpteroyldiglutamic acid (MTX-Glu₂), 4-amino-10-methylpteroyltriglutamic acid (MTX-Glu₃), 4-amino-10-methylpteroyltetraglutamic acid (MTX-Glu₄), 4-amino-10-methylpteroylpentaglutamic acid (MTX-Glu₅), 4-amino-10-methylpteroylhexaglutamic acid (MTX-Glu₆), and 4-amino-10-methylpteroylheptaglutamic acid (MTX-Glu₇) were purchased from Schircks Laboratories (Jona, Switzerland). All peptides listed in Supplementary Table 1 were synthesized by Thermo Fisher Scientific (Sedantrabe, Germany). Membrane vesicles prepared from MRP5-transfected and parental vector-transfected HEK293 cells were purchased from SOLVO Biotechnology (Budapest, Hungary). All other reagents were commercial products of analytical grade.

Cell culture

Six human stomach cancer cell lines (St-4, MKN45, MKN1, MKN28, MKN7, and MKN74) and five human breast cancer cell lines (MCF-7, HBC-4, BSY-1, HBC-5, and MDA-MB-231) in a panel of human cancer cell lines (JFCR-39) (Yamori, 2003) were seeded on non-coated tissue culture dishes (BD Biosciences, Bedford, MA) and cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA) containing 5% inactivated fetal bovine serum (FBS), penicillin (70 μg/mL), and streptomycin (100 μg/mL) at 37°C with an atmosphere of 5% CO₂ in air.
Preparation of membrane fraction

The cells (1.0 x 10^7 cells) were suspended in buffer containing 250 mM sucrose, 10 mM Tris-HCl (pH 7.4) and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and lysed by nitrogen cavitation at 450 psi for 15 min at 4°C. Lysed cells were centrifuged at 10,000 g for 10 min at 4°C and the supernatants were collected. They were centrifuged at 100,000 g for 60 min at 4°C. The resulting pellet was suspended in 250 mM sucrose, 10 mM Tris-HCl (pH 7.4) and stored as membrane fraction. Protein concentrations were measured by the Lowry method using the DC protein assay reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Average recovered amount of membrane proteins from the 11 cell lines was 516 µg protein/1.0 x 10^7 cells (247-863 µg protein/1.0 x 10^7 cells).

Protein quantification by multiplexed SRM/MRM analysis with LC-MS/MS

The absolute amounts of membrane proteins and metabolic enzymes of methotrexate were determined by using a multiplexed SRM/MRM method as described before (Kamiie et al., 2008). Membrane fraction or whole cell lysate (at least 50 µg protein) was suspended in 0.5 M Tris-HCl (pH 8.5), 7 M guanidine hydrochloride, 10 mM EDTA, and the proteins were S-carbamoylmethylated. The alkylated proteins were precipitated with a mixture of methanol and chloroform. The precipitates were dissolved in 6 M urea, diluted with 100 mM Tris-HCl (pH 8.5) and digested with sequence grade-modified trypsin (Promega, Madison, USA) at an enzyme/substrate ratio of 1:100 at 37°C for 16 hr. The tryptic digests were mixed with stable isotope-labeled peptide mixture as internal standard peptides. The tryptic digests were acidified with formic
acid, and then centrifuged at 15,000 rpm for 5 min at 4°C. The supernatants were injected into the high-performance liquid chromatography (HPLC) system (Agilent 1100 system; Agilent, Santa Clara, CA), which was connected to an ESI-triple quadrupole mass spectrometer (API5000; AB Sciex, Foster City, CA). HPLC was performed with C18 columns (ZORBAX SB-C18, 0.5 mm ID x 150 mm, 5 µm particles; Agilent). Linear gradients of 1-50% acetonitrile in 0.1% formic acid were applied to elute the peptides at a flow rate of 50 µL/min for 50 min. The mass spectrometer was set up to run a SRM/MRM experiment for peptide detection, using a dwell time of 10 msec per SRM/MRM transition. The ion counts in the chromatograms were determined by using the quantitation procedures in Analyst software version 1.5 (AB Sciex).

In the SRM/MRM analysis, each peptide for a target protein was monitored with four kinds of SRM/MRM transitions specific for that peptide. The quantitative value was calculated from the peak area ratio of analyte and stable isotope-labeled peptide in each SRM/MRM transition. Unless otherwise mentioned, at least three of four SRM/MRM transitions were required to be measurable for a proteotypic peptide to be judged as confirmed, and for a quantitative value to be assigned. The value of quantification limit of each protein (fmol/µg protein) was determined as described previously (Kawakami et al., 2011).

[3H]MTX uptake study

Five human breast cancer cell lines (MCF-7, HBC-4, BSY-1, HBC-5, and MDA-MB-231) were seeded on non-coated 24-well plates (Corning, Corning, NY) and cultured for 48 hr. The cells were washed with uptake buffer (122 mM NaCl, 25 mM
NaHCO₃, 10 mM D-glucose, 3 mM KCl, 1.4 mM CaCl₂, 1.2 mM MgSO₄·7H₂O, 0.4 mM K₂PO₄, 10 mM HEPES, pH 7.4), and the uptake study was initiated at 37°C by applying 200 μL uptake buffer containing 0.5 μCi [³H]MTX (90 nM). After a predetermined interval, cells were washed with ice-cold uptake buffer to terminate the uptake and solubilized. Radioactivity was measured in a liquid scintillation counter (LS6500, Beckman-Coulter, Fullerton, CA) and the protein content was determined by the Lowry method using the DC protein assay reagent (Bio-Rad) with bovine serum albumin as a standard.

Etoposide and MTX uptake study with SRM/MRM analysis by LC-MS/MS

Three human stomach cancer cell lines (St-4, MKN45, and MKN1) for etoposide uptake and two human breast cancer cell lines (HBC-4 and HBC-5) for MTX uptake were seeded on non-coated 24-well plates (Corning) or non-coated 12-well plates (Corning) and cultured for 48 hr. The cells were washed with uptake buffer (122 mM NaCl, 25 mM NaHCO₃, 10 mM D-glucose, 3 mM KCl, 1.4 mM CaCl₂, 1.2 mM MgSO₄·7H₂O, 0.4 mM K₂PO₄, 10 mM HEPES, pH 7.4), and the uptake study was initiated at 37°C by applying 1 mL RPMI1640 medium containing 1 μM etoposide or 200 nM MTX. In the etoposide uptake study, 50 μM MK571 was used as a MRPs inhibitor. MK571 was reported to inhibit MRP1-mediated transport of LTC4 and GSSG, MRP1 substrates, with a Ki value of 0.6 μM (Leier et al., 1996; Leier et al., 1994). After a predetermined interval, cells were washed with ice-cold uptake buffer to terminate the uptake and solubilized with 0.02 M NaOH or buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, and 1.5 mM MgCl₂. An equal volume of 0.1% formic acid in acetonitrile was added to remove proteins. These samples were acidified with formic acid.
acid, and then centrifuged at 15,000 rpm for 5 min at 4°C. The supernatants were dried by centrifugation under vacuum, solubilized by adding 0.1% formic acid in water, and then centrifuged at 15,000 rpm for 5 min at 4°C. The supernatants were subjected to LC-MS/MS analysis. The conditions of LC-MS/MS analysis are given in Supplementary Table 2.

Steady-state cell-to-medium (C/Mss, μL/mg protein) ratio was calculated as the ratio of intracellular drug amount per cellular protein amount to drug concentration in the medium, and is also described by Eq. 1 in terms of permeability clearance.

\[
\text{C/Mss ratio} = \frac{\text{PS}_{\text{inf}} \times V_{\text{cell}}}{(\text{PS}_{\text{eff, MK insensitive}} + \text{PS}_{\text{eff, MK sensitive}})} \quad \text{Eq. 1}
\]

Where \( V_{\text{cell}} \), \( \text{PS}_{\text{inf}} \), \( \text{PS}_{\text{eff, MK insensitive}} \) and \( \text{PS}_{\text{eff, MK sensitive}} \) represent volume of cells, influx clearance, MK571-insensitive efflux clearance and MK571-sensitive efflux clearance. The C/Mss ratio in the presence of MK571 (C/Mss ratio,MK571) is thus equal to \( \frac{\text{PS}_{\text{inf}} \times V_{\text{cell}}}{\text{PS}_{\text{eff, MK insensitive}}} \). From these equations, Eq. 2 can be obtained.

\[
\frac{1}{(\text{C/Mss ratio})} - \frac{1}{(\text{C/Mss ratio,MK571})} = \frac{\text{PS}_{\text{eff, MK sensitive}}}{(\text{PS}_{\text{inf}} \times V_{\text{cell}})} \quad \text{Eq. 2}
\]

With the assumption that \( (\text{PS}_{\text{inf}} \times V_{\text{cell}}) \) is constant, \( \frac{1}{(\text{C/Mss ratio})} - \frac{1}{(\text{C/Mss ratio,MK571})} \) is proportional to \( \text{PS}_{\text{eff, MK sensitive}} \). The derivation of Eq. 1 and 2 is shown in detail in Supplementary Information 1.

Vesicular uptake study of gemcitabine and MTX with SRM/MRM analysis by UPLC-MS/MS
The protein amount of membrane vesicles was determined by the Lowry method using the DC protein assay reagent (Bio-Rad) with bovine serum albumin as a standard. Uptake of gemcitabine and MTX by membrane vesicles was determined by the rapid filtration method, as described previously (Uchida et al., 2007). Briefly, vesicular uptake was done in uptake medium (250 mM sucrose, 10 mM Tris-HCl, 10 mM MgCl₂, 4 mM ATP, 10 mM phosphocreatine, 100 μg/mL creatine phosphokinase, pH 7.4) containing 100 μM gemcitabine or 10 μM MTX (a known substrate of MRP5). The reactions were carried out at 37°C for 10 min (for gemcitabine) or 3 min (for MTX), and stopped by the addition of ice-cold stop solution (250 mM sucrose, 10 mM Tris-HCl, 100 mM NaCl, pH 7.4). The samples were passed through 0.22 μm Durapore membrane filters (Millipore, Bedford, MA) under vacuum. The filters were then washed three times with ice-cold stop solution, and extracted with methanol at room temperature for 1 hr. The extracts were concentrated by centrifugation under vacuum. Then 0.1% formic acid in water was added to the concentrated extracts. This sample was subjected to UPLC-MS/MS analysis. The conditions of UPLC-MS/MS analysis are given in Supplementary Table 2. Uptake amount of gemcitabine or MTX was measured by UPLC-MS/MS and expressed as vesicle-to-medium ratio (μL/mg protein).

Cytotoxicity assay

The cytotoxicity was assessed by colorimetric assay using a Cell Counting Kit (Dojindo, Kumamoto, Japan). The cells were seeded on 96-well plates (Nunc, Roskilde, Denmark) in RPMI1640 medium. After 24 hr, cells were incubated with several concentrations of etoposide (0.1-100 μM) and 50 μM MK571 (MRPs inhibitor) for 72 hr, and then the cell viability was determined according to the manufacturer’s
instructions. Absorbance was measured at 450 nm with a microplate reader (Model 680, Bio-Rad). The IC<sub>50</sub> was estimated by plotting the rate of cell viability versus the drug concentration.

**SDS-PAGE and in-gel digestion**

Whole cell lysates of two human breast cancer cell lines (HBC-4 and HBC-5) were mixed with sample buffer, incubated at 37°C for 1 hr and loaded onto 12% SDS-PAGE gels. Proteins were visualized by Coomassie blue staining. The gel bands that contained FPGS protein (expected molecular weight; 65 kDa) were cut out and minced into small pieces (approximately 1.0 mm<sup>2</sup>). These gel pieces were washed with 500 μL 50 mM NH₄HCO₃ in 50% methanol for 15 min and centrifuged. Then, 500 μL 50 mM NH₄HCO₃ in 50% acetonitrile was added to the gel pieces and centrifuged. This procedure was repeated until no visible Coomassie blue stain remained. Then, 100 μL acetonitrile was added and the mixture was centrifuged. The remaining gel pieces were reduced by the addition of 10 mM dithiothreitol in 6 M guanidine hydrochloride/50 mM NH₄HCO₃ and incubated for 1 hr at room temperature. Acetonitrile (300 μL) was added and the mixture was centrifuged. The gel pieces were then dried and alkylated at room temperature in the dark for 45 min by the addition of 50 mM iodoacetamide in 6 M guanidine hydrochloride/50 mM NH₄HCO₃. After removal of the liquid, a solution containing equal volumes of 10% acetic acid and 100% methanol was added to the gel pieces, and the sample tubes were shaken for 30 min. This procedure was repeated up to three times. Then, 300 μL of 50 mM NH₄HCO₃ was added to the gel pieces, and the sample tubes were shaken for 15 min. After removal of the liquid, 300 μL acetonitrile was again added to the gel pieces. The sample tubes were shaken for 15 min, and then
the gel pieces were completely dried in a speed vacuum evaporator for 10 min. Trypsin solution (sequence-grade modified trypsin) was added to the gel pieces and incubated at 37°C for 16 hr. The gel pieces were extracted with 0.1% formic acid in 66% acetonitrile by sonication for 10 min. The supernatant was stored, and this procedure was repeated up to two times. The remaining gel pieces were further extracted with acetonitrile for 15 min. The supernatant was combined and concentrated to approximately 50 μL. The concentrated solution (trypsin-digested peptides) was mixed with stable isotope-labeled peptide mixture as internal standard peptides. The tryptic digests were acidified with formic acid, and then centrifuged at 15,000 rpm for 5 min at 4°C. The samples were subjected to LC-MS/MS analysis.
Results

Protein expression levels of membrane proteins in human stomach and breast cancer cell lines.

The expression amounts of 90 membrane proteins, i.e., 34 ABC transporter proteins, 53 SLC transporter proteins, 2 membrane marker proteins (Na⁺/K⁺ ATPase and γ-gtp) and 1 other membrane molecule (RLIP76), were quantified in membrane fraction of six human stomach cancer cell lines and five human breast cancer cell lines by means of quantitative targeted absolute proteomics (QTAP). Eleven membrane proteins (MRP1, GLUT1, 4F2hc, LAT1, MCT1, RFC1, ENT1, ATA1, PCFT, Na⁺/K⁺ ATPase and γ-gtp) were detected in the human stomach cancer cell lines (Table 1). These 11 proteins, together with MRP5, BCRP, and OATP3A1, were detected in the human breast cancer cell lines (Table 2). GLUT1, 4F2hc and Na⁺/K⁺ ATPase were detected in all 11 cell lines, while BCRP expression was detected only in HBC-5 human breast cancer cell line. The other 76 membrane proteins, including MDR1, were all under the limit of quantification in all of the cell lines (Table 3).

Correlation of MRP1 protein expression level to chemosensitivity of human stomach cancer cell lines.

MRP1 has been reported to transport various anti-cancer drugs, such as vincristine, doxorubicin, epirubicin and methotrexate (Garrido et al., 2011; Nunoya et al., 2003; Zeng et al., 2001), and it was detected in 3 out of 6 stomach cancer cell lines, but in only 1 out of 5 breast cancer cell lines. Among the stomach cancer cell lines, more than 16.2-fold difference was observed in the expression level of MRP1, which was the second-largest difference after that of glucose transporter GLUT1 expression (19.3-fold...
Among the 3 stomach cancer cell lines expressing MRP1 (St-4, MKN45 and MKN1), the protein expression levels of MRP1 in membrane fraction inversely correlated with sensitivity ($|\log_{10} GI_{50}|$) to etoposide (R=-0.994), vincristine (R=-0.994), epirubicin (R=-0.986), vinblastine (R=-0.964) and doxorubicin (R=-0.952) (Fig. 1). $|\log_{10} GI_{50}|$ values were obtained from a database of chemosensitivities of a panel of human cancer cell lines (Nakatsu et al., 2005). High GLUT1 protein expression (> 149 fmol/µg protein of membrane fraction) was detected in the three stomach cancer cell lines in which MRP1 was not detected (MKN28, MKN7 and MKN74), while the MRP1-expressing cell lines showed lower levels of GLUT1 protein expression (14.8-55.0 fmol/µg protein of membrane fraction) (Table 1).

**Involvement of MRP1 in etoposide sensitivity of human stomach cancer cell lines.**

Among the anti-cancer drugs shown in Fig. 1, etoposide has been used for treatment of stomach cancer (Lage, 2003; Morant, 2001). The involvement of MRP1 in etoposide sensitivity was examined by cytotoxicity assay using the three MRP1-expressing stomach cancer cell lines (St-4, MKN45 and MKN1). In the presence of 50 µM MK571, used for a MRPs inhibitor, the IC$_{50}$ value of etoposide was decreased in all three cell lines (Table 4). The decreases of the IC$_{50}$ values in the high MRP1-expressing cell lines (St-4 and MKN45) were greater than that in the lower MRP1-expressing cell line (MKN1).

In order to examine whether MRP1 protein expression level influences the intracellular amount of etoposide, cell-to-medium ratio of etoposide under a steady-state condition was measured in the presence or absence of MK571. In the presence of 50 µM
MK571, the cell-to-medium ratio of etoposide was increased in all three stomach cancer cell lines (Fig. 2A). In addition, the values of $1/(C/M_{ss} \text{ ratio}) - 1/(C/M_{ss} \text{ ratio, MK571})$, which reflects MK571-sensitive efflux clearance of etoposide, as described in Materials and Methods, correlated well with the protein expression levels of MRP1 ($R=0.998$) (Fig. 2B).

**Correlation of protein expression levels of membrane proteins to anti-cancer drug sensitivity in human breast cancer cell lines.**

The database on chemosensitivity of the panel of human cancer cell lines showed that there were large differences in sensitivity to gemcitabine and methotrexate (MTX) among the five breast cancer cell lines (1.00 x $10^4$-fold and 3.39 x $10^3$-fold differences in sensitivity to gemcitabine and MTX, respectively). As shown in Fig. 3, comparison of drug sensitivities and expression levels of transporter proteins in the five cell lines showed high correlation coefficients ($R$) between sensitivity to gemcitabine and the expression levels of 4F2hc ($R=0.781$) and MRP5 ($R=0.721$) (Fig. 3A and B). However, despite showing the highest $R$ value, 4F2hc was highly expressed only in MCF-7 cell line, and levels in the other four cell lines were low (Fig. 3A). In addition, it has been reported that 4F2hc itself lacks transport activity, and forms heterodimers with other amino acid transporters such as LAT1 and xCT (Ganapathy et al., 2009). Therefore, 4F2hc is likely to be indirectly involved in gemcitabine resistance.

Relatively high $R$ values were also observed between sensitivity to MTX and expression levels of RFC1 ($R=0.690$) and ENT1 ($R=0.566$) (Fig. 3C and D). As shown in Fig. 3C, a high correlation of the protein expression level of RFC1 with sensitivity ($|\log_{10} GI_{50}|$) to MTX ($R=0.965$) was observed for four breast cancer cell lines, excluding
HBC-5. This result suggests that RFC1 plays a major role in MTX sensitivity in these cell lines (MCF-7, HBC-4, BSY-1 and MDA-MB-231), whereas some other mechanism is likely to be involved in HBC-5.

**Involvement of MRP5 in gemcitabine transport and sensitivity**

Since protein expression level of MRP5 in the breast cancer cell lines was correlated to gemcitabine sensitivity (Fig. 3B), the transport of gemcitabine by MRP5 was examined by vesicular uptake study using MRP5-expressing membrane vesicles. The vesicle-to-medium ratio of gemcitabine in MRP5-expressing vesicles was not significantly different from that of mock vesicles after 10 min incubation (Fig. 4A). On the other hand, the vesicle-to-medium ratio of MTX, which is known to be a substrate of MRP5 (Wielinga et al., 2005), in MRP5-expressing vesicles was significantly greater (3.36-fold) than that of mock vesicles after 3 min incubation (Fig. 4B). This result suggests that gemcitabine is not a substrate of MRP5, though we cannot rule out the possibility that MRP5-mediated transport of other compounds may influence gemcitabine sensitivity.

**Involvement of RFC1 in methotrexate sensitivity in human breast cancer cell lines.**

The involvement of RFC1 in MTX sensitivity was examined by cellular uptake study of [³H]MTX using the breast cancer cell lines. [³H]MTX was taken up into each cell line in a time-dependent manner (data not shown). Initial uptake rate of [³H]MTX was directly correlated with the protein expression level of RFC1 and sensitivity to MTX (R=0.861 and R=0.956, respectively, excluding HBC-5) (Fig. 5). Furthermore, the cell-to-medium ratio of [³H]MTX at 48 hr incubation was directly correlated with the
expression level of RFC1 and sensitivity to MTX (R=0.982 and R=0.919, respectively, excluding HBC-5) (Fig. 6). The HBC-5 cell line showed a relatively high cell-to-medium ratio of [3H]MTX (202 μL/mg protein), even though it was resistant to MTX.

Protein expression levels of methotrexate-metabolizing enzymes in human breast cancer cell lines.

Since the MTX resistance of HBC-5 cell line could not be explained in terms of transporter protein expression, we considered that MTX metabolism might be involved. Therefore, protein expression amounts of MTX-metabolizing enzymes, dihydrofolate reductase (DHFR), folylpolyglutamate synthase (FPGS) and γ-glutamyl hydrolase (GGH), were quantified in whole cell lysate of MTX-sensitive HBC-4 and MTX-resistant HBC-5, since the expression level of RFC1 and uptake amount of [3H]MTX at 48 hr were similar in the two cell lines. Protein expression of DHFR and GGH was detected in both cell lines, but there was no significant difference in their expression levels between the two cell lines (Table 5). The protein expression of FPGS was under the limit of quantification in both cell lines. In order to reduce the background noise in QTAP analysis, whole cell lysates of these cell lines were separated by SDS-PAGE and in-gel-digested with trypsin. As a result of in-gel digestion, expression of FPGS was detected in both cell lines (Table 5). The expression level of FPGS in MTX-sensitive HBC-4 cell line was 2.38-fold greater than that in MTX-resistant HBC-5 cell line.

Intracellular amounts of methotrexate and methotrexate polyglutamates in human
breast cancer cell lines.

FPGS conjugates glutamate to MTX, producing MTX polyglutamates (MTX-Glu<sub>n</sub>) in the cells. Therefore, the cell-to-medium ratios of MTX and MTX polyglutamates (MTX-Glu<sub>2</sub> ~ MTX-Glu<sub>7</sub>) after incubation with MTX for 24 hr were measured. MTX and MTX polyglutamates (MTX-Glu<sub>2</sub> ~ MTX-Glu<sub>5</sub>) were detected in both HBC-4 and HBC-5 cell lines, while MTX-Glu<sub>6</sub> was detected only in HBC-4 cell line (Fig. 7A). MTX-Glu<sub>7</sub> was under the limit of quantification in both cell lines. The cell-to-medium ratio of MTX in MTX-resistant HBC-5 cell line was 2.01-fold greater than that in MTX-sensitive HBC-4 cell line, while the cell-to-medium ratios of MTX-Glu<sub>4</sub> ~ MTX-Glu<sub>6</sub> were greater in HBC-4 cell line (Fig. 7B).
Discussion

This is the first study to determine the absolute expression levels of multiple transporter proteins in membrane fraction of human stomach and breast cancer cell lines in order to identify key transporters involved in drug sensitivity. Among 90 membrane proteins examined, only GLUT1 and 4F2hc were present at quantifiable levels in all 11 cell lines, and RFC1 was quantifiable in 9 cell lines (Tables 1 and 2), indicating that protein expression levels vary widely from cell line to cell line, not only for xenobiotic transporters, but also for amino acid or nucleoside transporters. ENT1 was detected in all 5 breast cancer cell lines, but only 1 out of 6 stomach cancer cell lines. In contrast, MCT1 was not detected in breast cancer cell lines, but was detected in 3 stomach cancer cell lines. Previous studies have found greater ENT1 mRNA expression in breast cancer than in cancer cell lines derived from stomach cancer (Lu et al., 2002; Kameyama et al., 2011). Immunohistochemical analysis showed that the frequency of MCT1 expression was greater in human stomach cancer tissues than in breast cancer tissues (Pinheiro et al., 2009; Pinheiro et al., 2010). These transporter expression profiles are consistent with the present results.

MDR1 is an important ABC transporter involved in drug resistance of cancer cells. However, none of the cell lines expressed MDR1 at a sufficiently high level for quantification, though other ABC transporters, such as MRP1, MRP5 and BCRP, were detected (Tables 1, 2 and 3). Le et al. reported that MDR1 was highly expressed in a doxorubicin-resistant HepG2 cell line compared with the parental cell line (Li et al., 2004). Abolhoda et al. also reported that MDR1 mRNA was increased after administration of doxorubicin in patients with unresectable sarcoma pulmonary metastases (Abolhoda et al., 1999). Thus, MDR1 was proposed to play an important
role in acquisition of resistance to anti-cancer drugs by chemotherapy. Indeed, the human cancer cell lines used in this study had not acquired resistance via drug exposure.

As shown in Fig. 1, the protein expression level of MRP1 in membrane fraction was inversely correlated with sensitivity to five anti-cancer drugs in the three stomach cancer cell lines expressing MRP1. These five drugs were all reported to be substrates for MRP1 (Szakacs et al., 2006; Loscher and Potschka, 2005). Therefore, it seems likely that these anti-cancer drugs were pumped out from cells by MRP1 in these cell lines. The sensitivity to etoposide was highly correlated with MRP1 expression level, and sensitivity to etoposide was increased when MRP1 transport activity was inhibited with MK571 (Fig. 1 and Table 4). Although MK571 was reported to inhibit transport mediated by MRP4 and MRP5, as well as MRP1 (Reid et al., 2003), the present QTAP analysis showed that only MRP1 among the MK571-inhibited transporter proteins was expressed in these three stomach cancer cell lines (St-4, MKN45, and MKN1), suggesting that MK571 mainly inhibited efflux of etoposide mediated by MRP1.

In addition, the intracellular amount of etoposide was increased when MRP1 transport activity was inhibited in these cell lines (Fig. 2A). This result suggested that an increase of intracellular etoposide concentration accounts for the increase of sensitivity to etoposide under conditions of MRP1 inhibition (Table 4). Furthermore, the value of $1/(C/M_{ss} \text{ ratio}) - 1/(C/M_{ss} \text{ ratio, MK571})$ is proportional to MK571-sensitive efflux clearance, as described in Materials and Methods. As shown in Fig. 2B, the expression level of MRP1 was correlated with $1/(C/M_{ss} \text{ ratio}) - 1/(C/M_{ss} \text{ ratio, MK571})$ value. Therefore, it appears that the expression level of MRP1 regulates etoposide efflux clearance in the cells. It has been reported that MRP1-transfected HEK293 cells showed increased resistance to etoposide compared to the parental cells (Nunoya et al., 2003).
Furthermore, T98G glioblastoma cells treated with tacrolimus, which decreases MRP1 expression, were reported to show increased sensitivity to etoposide (Garrido et al., 2011). These previous studies suggested the involvement of MRP1 in etoposide resistance, but did not determine the intracellular amount of etoposide. Our results indicate that a decrease of intracellular etoposide concentration owing to MRP1-mediated efflux of etoposide is involved in etoposide resistance of stomach cancer cell lines.

The present comparison between protein expression level and drug sensitivity suggested that the mechanism of drug sensitivity involves the target transporters in some of the cell lines, but not in others. In the case of MRP1 expression and etoposide sensitivity shown in Fig. 1A, three cell lines (St-4, MKN45 and MKN1) showed a good correlation, suggesting that they share a drug sensitivity mechanism involving MRP1. However, the other three lines (MKN28, MKN7 and MKN74) did not exhibit such a correlation, and mechanisms not involving MRP1 presumably influence the etoposide sensitivity. These three cell lines expressed GLUT1 at higher levels and were less sensitive to etoposide than the three MRP1-expressing cell lines. Overall survival of patients with high-GLUT1-expressing stomach cancer was reported to be shorter than that of patients with lower GLUT1 expression (Kawamura et al., 2001). Thus, high expression of GLUT1 could be involved in another mechanism of etoposide resistance associated with increased malignancy of stomach cancer cell lines.

In the case of RFC1 expression and MTX sensitivity shown in Fig. 3C, four cell lines (MCF-7, HBC-4, BSY-1 and MDA-MB-231) were suggested to share the drug sensitivity mechanism involving RFC1. RFC1 is an influx transporter of folate and also accepts MTX as a substrate (Sharif et al., 1998). The present study is the first to
demonstrate a correlation of protein expression level of RFC1 with MTX sensitivity and intracellular amount of [\textsuperscript{3}H]MTX. RFC1-mediated uptake of MTX is expected to be increased in breast cancer cell lines with increased RFC1 expression, resulting in increased sensitivity to MTX.

In contrast, HBC5 was not on the correlation line of the four cell lines, suggesting that some other mechanism(s) predominantly influences MTX sensitivity in HBC-5. BCRP was detected only in HBC-5 among the 11 cancer cell lines. BCRP belongs to the ABCG family and was reported to mediate efflux transport of MTX from cells (Volk and Schneider, 2003; Chen et al., 2003). Therefore, BCRP is likely to be involved in the MTX resistance of HBC-5 cell line. However, the MTX level in HBC-5 was greater than that in HBC-4 after exposure to MTX for 48 h, even though HBC-5 was more resistant than HBC-4. This result indicates that differences in transmembrane transport of MTX mediated by transporters cannot explain the difference in MTX sensitivity between HBC-5 and HBC-4.

Glutamate residues are added to MTX in cells, and efflux of MTX polyglutamates from the cells is slow, resulting in prolonged retention and enhanced efficacy of MTX (Cole et al., 2001). Decreased intracellular levels of MTX polyglutamates were reported to be involved in MTX resistance (Assaraf, 2007; Waltham et al., 1997). This polyglutamylation process is dependent on both FPGS, which adds glutamate residues to MTX, and GGH, which removes glutamate residues (Waltham et al., 1997). It was also reported that overexpression of DHFR, which is the target enzyme of MTX, was involved in MTX resistance (Gorlick et al., 1996). In HBC-5, the expression level of FPGS was only 42.1% relative to that in HBC4, while the levels of GGH and DHFR were not significantly different (Table 5). Furthermore, the amounts of MTX with
longer glutamate chains (MTX-Glu4, Glu5 and Glu6) were less in HBC-5 than in HBC4 (Fig. 7). Taking these results into consideration, the difference of FPGS protein expression was considered to be involved in the difference in MTX sensitivity between HBC-5 and HBC-4. However, the difference in intracellular level of even MTX-Glu6, the longest detected glutamate chain, was 2.09-fold (HBC-4: 199 fmol/mg protein vs HBC-5: U.L.Q. <95.1 fmol/mg protein), while HBC-5 was 1.29 x 10^3-fold more resistant to MTX as compared with HBC-4. Therefore, the difference of FPGS expression may not fully explain the difference in MTX resistance. The DHFR-inhibitory activities of individual MTX polyglutamates are unknown, and should be clarified in a future analysis. Furthermore, an effect of mutation in DHFR cannot be ruled out, because mutation of leucine 22 to arginine in DHFR lowered the affinity for MTX and was reported to be involved in MTX resistance (Lewis et al., 1995).

Our results indicate that a decreased intracellular concentration of etoposide owing to higher expression of MRP1 results in etoposide resistance in human stomach cancer cell lines. In human breast cancer cell lines, a decreased intracellular concentration of MTX owing to lower expression of RFC1 appeared to be involved in MTX resistance, together with decreased concentrations of intracellular MTX polyglutamates, owing to lower expression of FPGS. Moreover, the present correlation analysis indicates that multiple proteins are candidates for involvement in anti-cancer drug resistance. GLUT1 was suggested to be involved in etoposide resistance in stomach cancer cell lines, and MRP5 and 4F2hc were suggested to be involved in gemcitabine sensitivity in breast cancer cell lines. These findings suggest that absolute quantification of multiple membrane proteins by means of QTAP could be useful strategy for identification of candidate proteins involved in drug sensitivity in other cases.
Authorship Contributions

Participated in research design: Obuchi, Ohtsuki, Terasaki

Conducted experiments: Obuchi, Ohmine

Contributed new reagents or analytic tools: Obuchi, Uchida, Ohmine, Yamori

Performed data analysis: Obuchi, Ohtsuki

Wrote or contributed to the writing of the manuscript: Obuchi, Ohtsuki, Yamori, Terasaki
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18F-FLT uptake with equilibrative nucleoside transporter-1 and thymidine kinase-1 expressions in gastrointestinal cancer. *Nucl Med Commun* **32**:460-5.


Sharif KA, Moscow JA and Goldman ID (1998) Concentrating capacity of the human reduced folate carrier (hRFC1) in human ZR-75 breast cancer cell lines.


Footnotes

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Person to receive reprint requests

Prof. Sumio Ohtsuki, Department of Pharmaceutical Microbiology, Faculty of Life Sciences, Kumamoto University, 5-1 Oe-honmachi, Chuo-ku, Kumamoto 862-0973, Japan; Email: sohtsuki@kumamoto-u.ac.jp
Legend for Figures

Fig. 1 Correlation between protein expression level of MRP1 and drug sensitivity of six human stomach cancer cell lines

Chemosensitivity to anticancer drugs (etoposide, vincristine, epirubicin, vinblastine, doxorubicin) was obtained from a database of chemosensitivities of a panel of human cancer cell lines (Nakatsu et al., 2005). The GI50 value is 50% growth-inhibitory concentration. Each dot and bar on the Y-axis represents the mean ± S.E.M. (n=1-3).

The correlation coefficient (R) in this figure refers only to the three MRP1-expressing cell lines (St-4, MKN1, MKN45).

Fig. 2 Effect of MK571 on the uptake amount of etoposide in three human stomach cancer cell lines

Three human stomach cancer cell lines (St-4, MKN45, MKN1) were incubated at 37°C for 48 hours in uptake medium containing 1 μM etoposide with or without 50 μM MK571, a MRPs inhibitor. Uptake amount of etoposide was measured by HPLC-MS/MS and was expressed as cell-to-medium ratio (C/M ratio, μL/mg protein).

(A) Effect of MK571 on the uptake amount of etoposide in three human stomach cancer cell lines. Each column represents the mean ± S.E.M. (n=4). (B) Correlation between 1/(C/M ratio) - 1/(C/M ratio,MK571) and expression level of MRP1. Each dot and bar on the Y-axis represents the mean ± S.E.M. (n=3).

Fig. 3 Correlation between anti-cancer drug sensitivity and protein expression level
in five human breast cancer cell lines

Chemosensitivity to MTX and gemcitabine was obtained from a database of chemosensitivities of a panel of human cancer cell lines (Nakatsu et al., 2005). The GI\textsubscript{50} value is the 50\% growth-inhibitory concentration. Dots and bars on the Y-axis represent the mean ± S.E.M. (n=3).

**Fig. 4 Uptake of gemcitabine by MRP5-expressing membrane vesicles**

Membrane vesicles (10 µg) prepared from MRP5-transfected (MRP5, open column) or parental vector-transfected (Mock, closed column) HEK293 cells were incubated at 37°C for 10 min (for gemcitabine) or 3 min (for MTX, a known substrate for MRP5) in the uptake medium containing 100 µM gemcitabine or 10 µM MTX in the presence of 4 mM ATP. The uptake amount of gemcitabine or MTX was measured by UPLC-MS/MS and expressed as vesicle-to-medium ratio (µL/mg protein). Columns and bars represent the mean ± S.E.M. (n=4). *p<0.05

**Fig. 5 Correlation of initial uptake of \([3H]\)MTX to RFC1 protein level or sensitivity to MTX in five human breast cancer cell lines**

Five human breast cancer cell lines (MCF-7, HBC-4, BSY-1, HBC-5, MDA-MB-231) were incubated at 37°C for 3, 10, and 30 min in uptake medium containing 0.5 µCi/200 µL (90 nM) of \([3H]\)MTX. (A) Correlation between initial uptake rate of \([3H]\)MTX and RFC1 expression in five human breast cancer cell lines. Each dot and bar represent the mean ± S.E.M. (X-axis: n=3, Y-axis: n=4). (B) Correlation between initial uptake rate of \([3H]\)MTX and chemosensitivity to MTX. Chemosensitivity to MTX was obtained from a database of chemosensitivities of a panel of human cancer cell lines (Nakatsu et al.,...
2005). The GI_{50} value is 50% growth-inhibitory concentration. Each dot on the Y-axis represents the mean (n=4).

**Fig. 6 Correlation of intracellular amount of [\textsuperscript{3}H]MTX in the steady state to RFC1 protein expression level or MTX sensitivity of five human breast cancer cell lines**

Five human breast cancer cell lines (MCF-7, HBC-4, BSY-1, HBC-5, MDA-MB-231) were incubated at 37°C for 48 hours in uptake medium containing 0.5 μCi/1 mL (18 nM) of \([\textsuperscript{3}H]\)MTX. Intracellular amount of \([\textsuperscript{3}H]\)MTX was expressed as cell-to-medium ratio (μL/mg protein). (A) Correlation between intracellular amount of \([\textsuperscript{3}H]\)MTX and RFC1 protein expression level in a steady-state condition for five human breast cancer cell lines. Each dot and bar represent the mean ± S.E.M. (X-axis: n=3, Y-axis: n=4). (B) Correlation between intracellular amount of \([\textsuperscript{3}H]\)MTX and chemosensitivity to MTX in a steady-state condition. Chemosensitivity to MTX was obtained from a database of chemosensitivities of a panel of human cancer cell lines (Nakatsu et al., 2005). The GI_{50} value is 50% growth-inhibitory concentration. Each dot on the Y-axis represents the mean (n=4).

**Fig. 7 Intracellular accumulation of methotrexate polyglutamates in methotrexate-resistant and sensitive human breast cancer cell lines**

Two human breast cancer cell lines (HBC-4 and HBC-5) were incubated at 37°C for 24 hours in uptake medium containing 200 nM methotrexate. Intracellular amounts of methotrexate and its polyglutamates (MTX-Glu2~MTX-Glu6) were measured by LC-MS/MS. (A) Intracellular amounts of each MTX polyglutamate in HBC-4 and HBC-5 cell lines. Each dot and bar represent the mean ± S.E.M. (HBC-4: n=2, HBC-5: n=2).
n=3). (B) Relative intracellular amount of each MTX polyglutamate in MTX-resistant HBC-5 cell line as compared to MTX-sensitive HBC-4 cell line.
Table 1 Expression levels of membrane proteins in membrane fraction of six human stomach cancer cell lines.

<table>
<thead>
<tr>
<th></th>
<th>St-4</th>
<th>MKN45</th>
<th>MKN1</th>
<th>MKN28</th>
<th>MKN7</th>
<th>MKN74</th>
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<tr>
<td><strong>Quantitative values in stomach cancer cell lines (fmol/µg protein of membrane fraction)</strong></td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>ABCC1 / MRP1</td>
<td>2.45 ± 0.29</td>
<td>1.36 ± 0.13*</td>
<td>0.588 ± 0.072*</td>
<td>U.L.Q. (&lt;0.151)</td>
<td>U.L.Q. (&lt;0.156)</td>
<td>U.L.Q. (&lt;0.197)</td>
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<tr>
<td>ABC5 / MRP5</td>
<td>U.L.Q. (&lt;0.620)</td>
<td>U.L.Q. (&lt;0.756)</td>
<td>U.L.Q. (&lt;0.425)</td>
<td>U.L.Q. (&lt;0.235)</td>
<td>U.L.Q. (&lt;0.280)</td>
<td>U.L.Q. (&lt;0.445)</td>
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<tr>
<td>ABCG2 / BCRP</td>
<td>U.L.Q. (&lt;0.467)</td>
<td>U.L.Q. (&lt;0.311)</td>
<td>U.L.Q. (&lt;0.692)</td>
<td>U.L.Q. (&lt;0.706)</td>
<td>U.L.Q. (&lt;0.744)</td>
<td>U.L.Q. (&lt;0.972)</td>
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<tr>
<td><strong>SLC transporter proteins</strong></td>
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<td></td>
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<tr>
<td>SLC2A1 / GLUT1</td>
<td>55.0 ± 5.3</td>
<td>30.3 ± 2.7</td>
<td>14.8 ± 1.6</td>
<td>286 ± 10</td>
<td>149 ± 5</td>
<td>179 ± 12</td>
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<td>SLC3A2 / 4F2hc</td>
<td>21.5 ± 1.6</td>
<td>1.93 ± 0.25</td>
<td>5.49 ± 0.50</td>
<td>3.64 ± 0.14</td>
<td>2.23 ± 0.06</td>
<td>2.71 ± 0.22</td>
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<td>SLC7A5 / LAT1</td>
<td>2.93 ± 0.64*</td>
<td>U.L.Q. (&lt;0.242)</td>
<td>U.L.Q. (&lt;0.301)</td>
<td>U.L.Q. (&lt;0.255)</td>
<td>U.L.Q. (&lt;0.376)</td>
<td>U.L.Q. (&lt;0.356)</td>
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<td>SLC16A1 / MCT1</td>
<td>2.40 ± 0.46</td>
<td>0.929 ± 0.097*</td>
<td>2.73 ± 0.56*</td>
<td>U.L.Q. (&lt;0.846)</td>
<td>U.L.Q. (&lt;0.489)</td>
<td>U.L.Q. (&lt;0.580)</td>
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<tr>
<td>SLC19A1 / RFC1</td>
<td>0.659 ± 0.087</td>
<td>0.585 ± 0.141</td>
<td>0.389 ± 0.037*</td>
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<td>0.189 ± 0.053*</td>
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<td>SLC03A1 / OATP3A1</td>
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<td>U.L.Q. (&lt;0.334)</td>
<td>U.L.Q. (&lt;0.498)</td>
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<td>U.L.Q. (&lt;0.266)</td>
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<td>SLC29A1 / ENT1</td>
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<td>U.L.Q. (&lt;0.565)</td>
<td>U.L.Q. (&lt;0.596)</td>
<td>U.L.Q. (&lt;0.399)</td>
<td>U.L.Q. (&lt;0.311)</td>
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<td>SLC38A1 / ATA1</td>
<td>1.27 ± 0.39*</td>
<td>0.917 ± 0.126*</td>
<td>U.L.Q. (&lt;0.440)</td>
<td>0.724 ± 0.121*</td>
<td>1.98 ± 0.56*</td>
<td>U.L.Q. (&lt;0.317)</td>
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<tr>
<td>SLC46A1 / PCFT</td>
<td>1.60 ± 0.09*</td>
<td>0.513 ± 0.218*</td>
<td>U.L.Q. (&lt;0.744)</td>
<td>0.558 ± 0.048</td>
<td>0.400 ± 0.118*</td>
<td>U.L.Q. (&lt;0.242)</td>
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<td><strong>Membrane marker proteins</strong></td>
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</tr>
<tr>
<td>Na+/K+ ATPase</td>
<td>14.2 ± 1.7</td>
<td>8.61 ± 0.79</td>
<td>11.4 ± 1.5</td>
<td>10.6 ± 0.8</td>
<td>6.83 ± 0.51</td>
<td>4.28 ± 0.28</td>
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<tr>
<td>γ-gtp</td>
<td>1.31 ± 0.09*</td>
<td>0.907 ± 0.090*</td>
<td>U.L.Q. (&lt;0.356)</td>
<td>U.L.Q. (&lt;0.110)</td>
<td>U.L.Q. (&lt;0.0920)</td>
<td>U.L.Q. (&lt;0.117)</td>
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Membrane fraction of six human stomach cancer cell lines (St-4, MKN45, MKN1, MKN28, MKN7, MKN74) were analyzed by multiplexed-SRM/MRM analysis using LC-MS/MS. The expression amount of each molecule was determined as the average of 2 to 12 quantitative values from four SRM/MRM transitions in one to three analyses. Each value represents the mean ± S.E.M. (n=2-12 SRM/MRM transitions). U.L.Q. means “under the limit of quantification” and the value in brackets following U.L.Q. represents the value of the quantification limit (fmol/µg protein).

*The reliability of the calculated values is considered to be less than that of other
detected molecules, because in these cases only two among four SRM/MRM transitions gave detectable peak areas over 5000 counts.
Table 2 Expression levels of membrane proteins in membrane fraction of five human breast cancer cell lines.

<table>
<thead>
<tr>
<th></th>
<th>Quantitative values in breast cancer cell lines (fmol/µg protein of membrane fraction)</th>
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<tr>
<td></td>
<td>MCF-7</td>
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<tr>
<td><strong>ABC transporter proteins</strong></td>
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</tr>
<tr>
<td>ABC1 / MRP1</td>
<td>U.L.Q. (&lt;0.315)</td>
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<tr>
<td>ABC5 / MRP5</td>
<td>1.06 ± 0.16*</td>
</tr>
<tr>
<td>ABCG2 / BCRP</td>
<td>U.L.Q. (&lt;0.312)</td>
</tr>
<tr>
<td><strong>SLC transporter proteins</strong></td>
<td></td>
</tr>
<tr>
<td>SLC2A1 / GLUT1</td>
<td>18.7 ± 0.5</td>
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<tr>
<td>SLC3A2 / 4F2hc</td>
<td>62.2 ± 2.4</td>
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<td>SLC7A5 / LAT1</td>
<td>3.85 ± 0.65</td>
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<td>SLC16A1 / MCT1</td>
<td>U.L.Q. (&lt;0.445)</td>
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<td>SLC19A1 / RFC1</td>
<td>1.41 ± 0.33</td>
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<td>SLC29A1 / ENT1</td>
<td>3.28 ± 0.60</td>
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<td>SLC38A1 / ATA1</td>
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<td>SLC46A1 / PCFT</td>
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<td>Na(^+)/K(^+) ATPase</td>
<td>6.23 ± 0.42</td>
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<tr>
<td>(\gamma)-gtp</td>
<td>U.L.Q. (&lt;0.129)</td>
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Membrane fraction of five human breast cancer cell lines (MCF-7, HBC-4, BSY-1, HBC-5, MDA-MB-231) were analyzed by multiplexed-SRM/MRM analysis using LC-MS/MS. The expression amount of each molecule was determined as the average of 3 to 12 quantitative values from four SRM/MRM transitions in two or three analyses. Each value represents the mean ± S.E.M. (n=3-12 SRM/MRM transitions). U.L.Q. means “under the limit of quantification” and the value in brackets following U.L.Q. represents the value of the quantification limit (fmol/µg protein).

*The reliability of the calculated values is considered to be less than that of other
detected molecules, because in these cases only two among four SRM/MRM transitions gave detectable peak areas over 5000 counts.
Table 3 Membrane proteins under the limit of quantification in membrane fraction of six human stomach cancer cell lines and five human breast cancer cell lines

### ABC transporter proteins

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<thead>
<tr>
<th>ABCA1 / ABC1</th>
<th>ABCA2 / ABC2</th>
<th>ABCA3 / ABC3</th>
<th>ABCA4 / ABCR</th>
<th>ABCA5</th>
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<td>ABCA6</td>
<td>ABCA7</td>
<td>ABCA8</td>
<td>ABCA9</td>
<td>ABCA10 / ABC10</td>
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<tr>
<td>ABCA12 / ABC12</td>
<td>ABCB1 / MDR1</td>
<td>ABCB4 / MDR3</td>
<td>ABC5</td>
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<td>ABCC7 / CFTR</td>
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<td>ABCC9 / SUR2</td>
<td>ABCC10 / MRP7</td>
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### SLC transporter proteins

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<td>SLC15A2 / PEPT2</td>
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<td>SLC21A2 / PGT</td>
<td>SLC01A2 / OATP1A2</td>
<td>SLC01B1 / OATP1B1</td>
</tr>
<tr>
<td>SLC01B3 / OATP1B3</td>
<td>SLC01C1 / OATP1C1</td>
<td>SLC02B1 / OATP2B1</td>
<td>SLC04A1 / OATP4A1</td>
<td>SLC04C1 / OATP4C1</td>
</tr>
<tr>
<td>SLC22A9 / UST3</td>
<td>SLC22A10 / OAT5</td>
<td>SLC22A11 / OAT4</td>
<td>SLC22A12 / URAT1</td>
<td>SLC22A13 / OCTL1</td>
</tr>
<tr>
<td>SLC22A14 / OCTL2</td>
<td>SLC22A15 / FLIPT1</td>
<td>SLC22A16 / CT2</td>
<td>SLC22A18</td>
<td>SLC28A1 / CNT1</td>
</tr>
</tbody>
</table>

### Other molecules

RLIP76

Membrane fractions of six human stomach cancer cell lines (St-4, MKN45, MKN1, MKN28, MKN7, MKN74) and five human breast cancer cell lines (MCF-7, HBC-4, BSY-1, HBC-5, MDA-MB-231) were examined by multiplexed-SRM/MRM analysis using LC-MS/MS.
Table 4 Effect of MK571 on the chemosensitivity of three human stomach cancer cell lines to etoposide

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ (µM)</th>
<th>Control/MK571 ratio of IC₅₀</th>
<th>MRP1 expression (fmol/µg protein of membrane fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µM MK571</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>St-4</td>
<td>63.2</td>
<td>6.15</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.45 ± 0.29</td>
</tr>
<tr>
<td>MKN45</td>
<td>34.3</td>
<td>2.97</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.36 ± 0.13</td>
</tr>
<tr>
<td>MKN1</td>
<td>12.6</td>
<td>2.23</td>
<td>4.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.588 ± 0.072</td>
</tr>
</tbody>
</table>

Three human stomach cancer cell lines (St-4, MKN45, MKN1) were exposed for 72 hours to various concentrations of etoposide with or without 50 µM MK571, a MRPs inhibitor. Control was etoposide exposure without MK571. The IC₅₀ value was determined by plotting the percentage cell viability versus the concentration of etoposide. Each value of MRP1 expression represents the mean ± S.E.M. (n=4).
Table 5 Expression levels of metabolic enzymes of methotrexate in whole cell lysate of methotrexate resistant and sensitive human breast cancer cell lines

<table>
<thead>
<tr>
<th></th>
<th>Quantitative values (fmol/µg protein of cell lysate)</th>
<th>Expression ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HBC-4 (sensitive to MTX)</td>
<td>HBC-5 (resistant to MTX)</td>
</tr>
<tr>
<td>DHFR</td>
<td>0.859 ± 0.019</td>
<td>0.842 ± 0.026</td>
</tr>
<tr>
<td>FPGS</td>
<td>U.L.Q. (&lt; 0.327)</td>
<td>U.L.Q. (&lt; 0.330)</td>
</tr>
<tr>
<td>GGH</td>
<td>1.25 ± 0.06</td>
<td>1.18 ± 0.05</td>
</tr>
</tbody>
</table>

*In-gel digested sample*

<table>
<thead>
<tr>
<th></th>
<th>Quantitative values (fmol/µg protein of cell lysate)</th>
<th>Expression ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPGS</td>
<td>0.525 ± 0.170</td>
<td>0.221 ± 0.046</td>
</tr>
</tbody>
</table>

Whole cell lysates of two human breast cancer cell lines (HBC-4 and HBC-5) were digested with trypsin. Proteins of whole cell lysate for in-gel trypsin digestion were separated by SDS-PAGE, and the gel bands (50-75 kDa) were cut out for in-gel trypsin digestion. Tryptic digest of each cell line was analyzed by multiplexed-SRM/MRM with LC-MS/MS. The amount of each protein was determined as the average of 5 to 12 quantitative values from four SRM/MRM transitions in two or four analyses. Each value represents the mean ± S.E.M. (n=5-12 SRM/MRM transitions). U.L.Q. means “under the limit of quantification” and the value in brackets following U.L.Q. represents the value of the quantification limit (fmol/µg protein).
Fig. 1.

A. Etoposide

- **R** = -0.994 (only MRP1-expressing cells)

Expression level of MRP1 (fmoles/μg protein) vs. Sensitivity to etoposide (log_{10}GI_{50})

B. Vincristine

- **R** = -0.994 (only MRP1-expressing cells)

Expression level of MRP1 (fmoles/μg protein) vs. Sensitivity to vincristine (log_{10}GI_{50})

C. Epirubicin

- **R** = -0.986 (only MRP1-expressing cells)

Expression level of MRP1 (fmoles/μg protein) vs. Sensitivity to epirubicin (log_{10}GI_{50})

D. Vinblastine

- **R** = -0.964 (only MRP1-expressing cells)

Expression level of MRP1 (fmoles/μg protein) vs. Sensitivity to vinblastine (log_{10}GI_{50})

E. Doxorubicin

- **R** = -0.952 (only MRP1-expressing cells)

Expression level of MRP1 (fmoles/μg protein) vs. Sensitivity to doxorubicin (log_{10}GI_{50})
Fig. 2.

A

Cell to medium ratio (μL/mg protein)

- MK571 (-)
- 50 μM MK571

St-4  MKN45  MKN1

B

Expression level of MRP1 (fmol/μg protein)

$R = -0.998$

$1/(C/M\text{ ratio}) - 1/(C/M\text{ ratio}_{MK571})$

St-4  MKN45  MKN1
Fig. 3.

A. 4F2hc vs Gemcitabine

![Graph showing the relationship between 4F2hc expression level and sensitivity to gemcitabine.](image)

B. MRP5 vs Gemcitabine

![Graph showing the relationship between MRP5 expression level and sensitivity to gemcitabine.](image)

C. RFC1 vs Methotrexate

![Graph showing the relationship between RFC1 expression level and sensitivity to methotrexate.](image)

D. ENT1 vs Methotrexate

![Graph showing the relationship between ENT1 expression level and sensitivity to methotrexate.](image)
Fig. 7.

A

Uptake amount (fmol/mg protein)

MTX    MTX-Glu2    MTX-Glu3    MTX-Glu4    MTX-Glu5    MTX-Glu6

HBC-4: 

HBC-5: <95.1

B

Relative uptake amount

HBC-5 (resistant) / HBC-4 (sensitive)

MTX    MTX-Glu2    MTX-Glu3    MTX-Glu4    MTX-Glu5    MTX-Glu6

<0.477
Identification of transporters associated with etoposide sensitivity of stomach cancer cell lines and methotrexate sensitivity of breast cancer cell lines by quantitative targeted absolute proteomics

Wataru Obuchi, Sumio Ohtsuki, Yasuo Uchida, Ken Ohmine, Takao Yamori, Tetsuya Terasaki

Molecular Pharmacology
**Supplementary Table 1 Peptide probe sequences and selected ions for absolute quantification of membrane proteins and metabolic enzymes**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Alias</th>
<th>Probe sequence</th>
<th>SRM/MRM transition (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Q1</td>
</tr>
<tr>
<td>ABCA9</td>
<td>ABCA9</td>
<td>QHISDAK</td>
<td>399.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QHISDA*K</td>
<td>401.7</td>
</tr>
<tr>
<td>ABCB5</td>
<td>ABCB5</td>
<td>SADLIVTLK</td>
<td>480.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SADLIVTL*K</td>
<td>483.8</td>
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<tr>
<td>SLC16A3</td>
<td>MCT4</td>
<td>LLDLSVFR</td>
<td>482.4</td>
</tr>
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<td></td>
<td></td>
<td>LLDLSVF*R</td>
<td>487.4</td>
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<tr>
<td>SLC22A9</td>
<td>UST3</td>
<td>DTTLTEILK</td>
<td>523.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DTTLTEIL*K</td>
<td>526.9</td>
</tr>
<tr>
<td>SLC22A14</td>
<td>OCTL2</td>
<td>DQPLSESLNHSSQ*R</td>
<td>570.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DQPLSESLNHSSQI*R</td>
<td>573.2</td>
</tr>
<tr>
<td>SLC47A2</td>
<td>MATE2</td>
<td>YLQNQGWLK</td>
<td>575.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YLQNQGWL*K</td>
<td>578.8</td>
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<tr>
<td>SLC47A2</td>
<td>MATE2k</td>
<td>TPEEAHALSAPTS*R</td>
<td>733.9</td>
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<td>TPEEAHALSAAP*TSR</td>
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<td>DHFR</td>
<td>DHFR</td>
<td>NGDLPWPPLR</td>
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<td>NGDLPWPPL*K</td>
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<td>FPGS</td>
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<td>DIFQPPSPPK</td>
<td>611.8</td>
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<tr>
<td></td>
<td></td>
<td>DIFQPPSPPK*K</td>
<td>614.8</td>
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<tr>
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<td>GGH</td>
<td>YYIAASYVK</td>
<td>539.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YYIAASYVK*K</td>
<td>542.3</td>
</tr>
</tbody>
</table>

Typically, doubly charged precursor ions (singly charged for some peptides) were selected (Q1). Four transitions per peptide (Q3-1, -2, -3 and -4), corresponding to high-intensity fragment ions, were selected. Bold letters with asterisks indicate amino acid residues labeled with stable isotope (\(^{13}\text{C}\) and \(^{15}\text{N}\)). Other peptides were listed in previous reports (Ohtsuki et al., 2011; Uchida et al., 2011).


## Supplementary Table 2 Conditions of LC-MS/MS analysis in uptake studies

<table>
<thead>
<tr>
<th>Molecule</th>
<th>SRM/MRM transition (m/z)</th>
<th>DP (V)</th>
<th>CE (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q1</td>
<td>Q3</td>
<td></td>
</tr>
<tr>
<td>Etoposide</td>
<td>589.2</td>
<td>229.2</td>
<td>76</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>264.1</td>
<td>112.1</td>
<td>66</td>
</tr>
<tr>
<td>Methotrexate (MTX)</td>
<td>455.4</td>
<td>308.2</td>
<td>151</td>
</tr>
<tr>
<td>MTX-Glu₂</td>
<td>584.6</td>
<td>308.2</td>
<td>156</td>
</tr>
<tr>
<td>MTX-Glu₃</td>
<td>713.7</td>
<td>308.2</td>
<td>181</td>
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<tr>
<td>MTX-Glu₄</td>
<td>842.8</td>
<td>308.2</td>
<td>150</td>
</tr>
<tr>
<td>MTX-Glu₅</td>
<td>971.9</td>
<td>308.2</td>
<td>150</td>
</tr>
<tr>
<td>MTX-Glu₆</td>
<td>1101.0</td>
<td>308.2</td>
<td>150</td>
</tr>
<tr>
<td>MTX-Glu₇</td>
<td>1230.1</td>
<td>308.2</td>
<td>150</td>
</tr>
</tbody>
</table>
Supplementary Information 1. Derivation of Eq. 1 and 2

Based on the model shown in Fig S1A, medium-to-cell influx rate and cell-to-medium efflux rate are described as:

Medium-to-cell influx rate = $P_{\text{inf}} \times C_{\text{medium}}$  
Cell-to-medium efflux rate = $(P_{\text{eff,MK insensitive}} + P_{\text{eff,MK sensitive}}) \times C_{\text{cell}}$

Fig S1. Schematic diagram illustrating the PS products for the accumulation of substrates in human stomach cancer cells.

PS$_{\text{inf}}$, PS$_{\text{eff,MK insensitive}}$ and PS$_{\text{eff,MK sensitive}}$ represent influx clearance, MK571-insensitive efflux clearance and MK571-sensitive efflux clearance, respectively. $C_{\text{medium}}$ and $C_{\text{cell}}$ represent drug concentration in medium (nM) and cell (nmol/μg cellular protein), respectively. $V_{\text{cell}}$ represents volume of cells (μL).

Cell-to-medium (C/M) ratio represents the apparent distribution volume of the substrate in the cell generated by the polarized transport rate across the plasma membrane, and is defined as $(C_{\text{cell}} \times V_{\text{cell}}) / C_{\text{medium}}$. Considering the steady-state condition, medium-to-cell influx rate is equal to cell-to-medium efflux rate. Therefore, steady-state C/M$_{\text{ss}}$ ratio can be transformed to Eq.1 in the text using Supp Eq. 1 and 2.

$$P_{\text{inf}} \times C_{\text{medium}} = (P_{\text{eff,MK insensitive}} + P_{\text{eff,MK sensitive}}) \times C_{\text{cell}}$$

$$C/M_{\text{ss}} \text{ ratio} = (C_{\text{cell}} \times V_{\text{cell}}) / C_{\text{medium}} = P_{\text{inf}} \times V_{\text{cell}} / (P_{\text{eff,MK insensitive}} + P_{\text{eff,MK sensitive}})$$

Eq. 1 in the text

In the presence of MK571, PS$_{\text{eff,MK sensitive}}$ becomes zero, as shown in Fig S1B. Therefore, steady-state C/M$_{\text{ss}}$ ratio, MK571 can be described as:

$$C/M_{\text{ss}} \text{ ratio}, \text{MK571} = P_{\text{inf}} \times V_{\text{cell}} / P_{\text{eff,MK insensitive}}$$
Then, Eq. 2 in the text was derived as follows:

\[
\frac{1}{(C/M_{ss\text{ ratio}})} - \frac{1}{(C/M_{ss\text{ ratio},MK571})} = \frac{(PS_{\text{eff,MK insensitive}} + PS_{\text{eff,MK sensitive}})}{(PS_{\text{inf}} \times V_{cell})} - \frac{PS_{\text{eff,MK insensitive}}}{(PS_{\text{inf}} \times V_{cell})} \\
= \frac{PS_{\text{eff,MK sensitive}}}{(PS_{\text{inf}} \times V_{cell})}
\]

Eq. 2 in the text