TABLE III – KINETIC PARAMETERS FOR ATP-DEPENDENT TRANSPORT OF CPT ANALOGUES DETERMINED BY MEMBRANE VESICLE TRANSPORT

CPT analogues	PC-6			PC-6/SN2-5H2			HEK293			HEK293/[Lys141]ABCG2		
	Km	Vmax	V/K	Km	Vmax	V/K	Km	Vmax	V/K	Km	Vmax	V/K
SN-38	2.8	12.7	4.6	6.2	2000	322.6	3.8	5.9	1.6	2.3	400	175.4
SN-398	2.5	5.0	2.0	8.7	1563	178.9	3.7	3.6	1.0	1.4	200	147.1
SN-355	ND	ND	ND	5.7	769	135.1	ND	ND	ND	4.1	476	114.9
SN-392	12.5	15.8	1.3	8.3	1000	120.5	0.7	4.3	5.8	2.1	154	73.0

ATP-dependent transport of CPT analogues was measured with plasma membrane vesicles prepared from PC-6, PC-6/SN2-5H2, HEK293 and [Lys141]ABCG2-transfected HEK293 cells. Kinetic parameters such as the Michaelis-Menten constant (Km) and the maximum velocity (Vmax) were calculated from Lineweaver-Burk plots. The units of Km and Vmax value are μM and pmol/mg protein/min, respectively. ND indicates not detected.

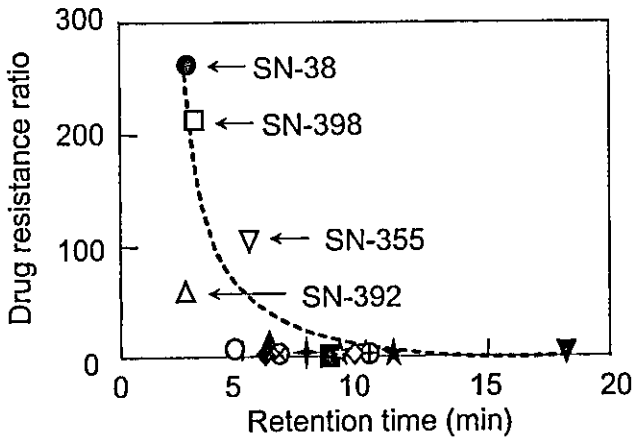

FIGURE 5 – Correlation between drug resistance ratio and polarity of CPT analogues. Retention time of CPT analogues was measured by HPLC. See Figure 1 for the symbols of CPT analogues. Drug resistance ratio is calculated from the ratio of IC_{50} (PC-6/SN2-5H2)/IC_{50} (PC-6).

with high polarity, *i.e.*, SN-355, SN-392 and SN-398, are closely related to ABCG2-associated drug resistance because they are extruded from tumor cells by ABCG2. To circumvent ABCG2-associated drug resistance, the present study strongly suggests that CPT analogues with low polarity would be good candidates for the molecular design of CPT-based antitumor drugs. Furthermore, it is important to mention that our SN-38 analogues with low polarity were not substrates of P-glycoprotein (ABCB1/MDR1/P-gp; data not shown).

CPT analogues and polymorphism of ABCG2

In the present study, we have cloned ABCG2 from PC-6/SN2-5H2 cells. The cloned cDNA encodes the ABCG2 protein with an arginine at amino acid 482, as does the wild type of ABCG2. However, the amino acid at position 141 of the cloned ABCG2 has been changed from Gln to Lys. This type of variation (Gln141Lys) has hitherto been reported as a naturally occurring SNP of ABCG2. The Gln141Lys polymorphism has recently been identified in healthy Japanese volunteers as well.[29] We investigated the effects of this SNP on the substrate specificity of ABCG2, using ABCG2-transfected cells with Gln at amino acid 141. The substitution of Lys141 to Gln141 did not affect the substrate specificity toward CPT analogues (data not shown).

The importance of ABC transporters in various diseases and biophylaxis has recently begun to be clarified. Molecular design based on the drug transport mechanisms of ABC transporters can improve the effectiveness of antitumor drugs and contribute to development of resistance-reversal drugs as well as new antitumor drugs. The transport mechanism-based molecular design strategy can also provide an effective tool for personalized chemotherapy against tumors. In this regard, we do hope that the CPT analogues tested in the present study would contribute to the molecular design of new antitumor drugs to cure the disease.

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Transport of SN-38 by the Wild Type of Human ABC Transporter ABCG2 and Its Inhibition by Quercetin, a Natural Flavonoid

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Irinotecan (CPT-11) is a widely-used potent anticancer drug that inhibits mammalian DNA topoisomerase I, however overexpression of the ATP-binding cassette transporter ABCG2 can confer cancer cells resistance to SN-38, the active form of CPT-11. In the present study, we have examined the contribution of three variant forms of ABCG2 to SN-38 resistance. Exogenous expression of the Arg482 (wild type), Gly482, and Thr482 variants of ABCG2 conferred HEK293 cells resistance to SN-38 by 15.0-, 5.0-, and 5.3-fold, respectively. In plasma membrane vesicles prepared from the ABCG2 variant cDNA-transfected HEK293 cells, [Arg482]ABCG2 transported SN-38 and its glucuronide conjugate in an ATP-dependent manner; however, only minimal transport activities were observed with the other variants (Gly482 and Thr482). In addition, we have screened natural flavonoids to find potent inhibitors of [Arg482]ABCG2. Quercetin was found to be the strongest inhibitor (Ki = 0.28 μM) among natural flavonoids tested in the plasma membrane system in this study. When [Arg482]ABCG2-transfected HEK293 cells were incubated with SN-38 in the presence of 20 μM quercetin, cellular resistance to SN-38 was partly reversed. In this context, certain flavonoid derivatives are considered to be good candidates for development of ABCG2 inhibitors.

Key words: ABC transporter, ABCG2, BCRP, irinotecan, multidrug resistance, flavonoid

Abbreviations: ABC, ATP-binding cassette; Irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin (CPT-11); SN-38, 7-ethyl-10-hydroxycamptothecin, SN-38glu, the glucuronide conjugate of SN-38; HEK, human embryonic kidney; IC_{50}, drug concentration required for 50%-inihibition; Ki, inhibition constant; MTT, bromo-3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium.

INTRODUCTION

Camptothecin, an alkaloidal anticancer drug, was originally isolated from the Chinese tree *Camptotheca acuminata* by Wall et al. (1) in 1966. Clinical and preclinical studies, however, revealed reversible bone marrow depression and hemorrhagic cystitis as the major dose-limiting toxicity of camptothecin (2,3). Thus, efforts have been directed at finding new camptothecin derivatives with higher anticancer activity and less toxicity. Kunimoto et al. (4) and Sawada et al. (5) have synthesized a potent and safer camptothecin derivative that is named irinotecan or

CPT-11. At present, CPT-11 is widely used for clinical chemotherapy of human cancer. CPT-11 is a prodrug whose anticancer activity is exerted by 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of CPT-11 (6). The molecular target of SN-38 is mammalian DNA topoisomerase I (Topo I). Like other camptothecin derivatives, SN-38 forms a Topo I-DNA-drug covalent complex to irreversibly inhibit the enzyme activity.

Despite enormous expense and efforts spent on the development of cancer chemotherapies, however, acquired and intrinsic drug resistance in cancer is the major obstacle to long-term, sustained patient response to chemotherapy. There is accumulating evidence that the active export of anticancer drugs from cancer cells is one of the major mechanisms of drug resistance. Several ATP-binding cassette (ABC) transporters underlie multidrug resistance in cancer cells by actively extruding the clinically administered chemotherapeutic drugs (7).

It has recently been reported that overexpression of ABCG2 confers cancer cells resistance to camptothecin-based anticancer drugs (8-10). In a previous study, SN-38-selected PC-6/SN2-5H human lung carcinoma cells were shown to overexpress ABCG2 with the reduced intracellular accumulation of SN-38 and its glucuronide metabolite (11). We have recently demonstrated that plasma membrane vesicles prepared from those cells ATP-dependently transported both SN-38 and SN-38-glucuronide, and our results strongly suggested that ABCG2 is involved in the active extrusion of SN-38 and its metabolite from cancer cells (12).

To date, at least three variant forms of ABCG2 have been documented on the basis of amino acid moieties at position 482, which resides within the third transmembrane domain. The wild-type form of ABCG2 has an arginine (Arg) at that position (13), whereas other variants cloned from cancer cell lines (14,15) have glycine (Gly) and threonine (Thr) at position 482. Our group and others have recently demonstrated that the substrate specificities and resistance profiles of ABCG2 greatly differ among those variant forms (16-20). In the present study, to elucidate the role of ABCG2 variants in the cellular resistance to SN-38, we have expressed each variant form of ABCG2 in HEK293 cells and examined their transport activity and substrate specificity toward SN-38 and SN-38glu. In this report, we provide direct evidence that [Arg482]ABCG2 transports SN-38.

In addition, inhibitors of [Arg482]ABCG2 are of great interest as chemosensitizers for clinical drug resistance to improve the pharmacokinetic profile of chemotherapeutic drugs. In this study, we have screened natural flavonoids to discover potent inhibitors of [Arg482]ABCG2. Quercetin was found to be the strongest inhibitor among natural flavonoids tested.

MATERIALS AND METHODS

Chemicals

SN-38 lactone and SN-38glu were provided by Yakult Honsha Co., Ltd. (Tokyo, Japan). SN-38 carboxylate was prepared by incubating SN-38 lactone with 100 mM NaOH at 4°C for 30 min. Mitoxantrone, doxorubicin, daunorubicin, vincristine, methotrexate, and cisplatin were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Paclitaxel (taxol) and etoposide were from CALBIOCHEM (Schwalbach, Germany). Mitomycin C was obtained from Kyowa Hakko Kogyo, Ltd. (Tokyo, Japan). Quercetin, theaflavine, theaflavine 3-*O*-gallate, catechin, and epigallocatechin, and epigallocatechin gallate were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical grade.

Cell lines

HEK293 cells were transfected with the pcDNA3.1 vector carrying the ABCG2 cDNA as described previously (18). Single colonies resistant to G418 (Nacalai Tesque, Kyoto, Japan) were picked and sub-cultured. HEK293 transfectants were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), penicillin (100 μ/ml), and streptomycin (100 mg/ml) in a humidified atmosphere of 5% CO_2 in air. The number of viable cells was determined in a hemocytometer by Trypan Blue dye exclusion.

Immuno-fluorescence microscopy

Mock- and ABCG2-transfected HEK293 cells were seeded on microscopic cover glasses and incubated under the above-mentioned culture conditions over night. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature. Thereafter, cell membranes were permeabilized by incubating the cells with 0.1% Triton X100 in PBS at room temperature for 5 min. Cells were then treated with the BXP-21 antibody (1:20 dilution; SIGNET, Dedham, MA) as the first antibody and subsequently with the Cy3-conjugated anti-mouse IgG antibody (1:500 dilution; Jackson ImmunoResearch Laboratories, Inc., Baltimore, MA). The immuno-fluorescence of HEK293 cells was detected with a confocal laser-scanning fluorescence microscope IX70/Fluoview (Olympus, Tokyo, Japan).

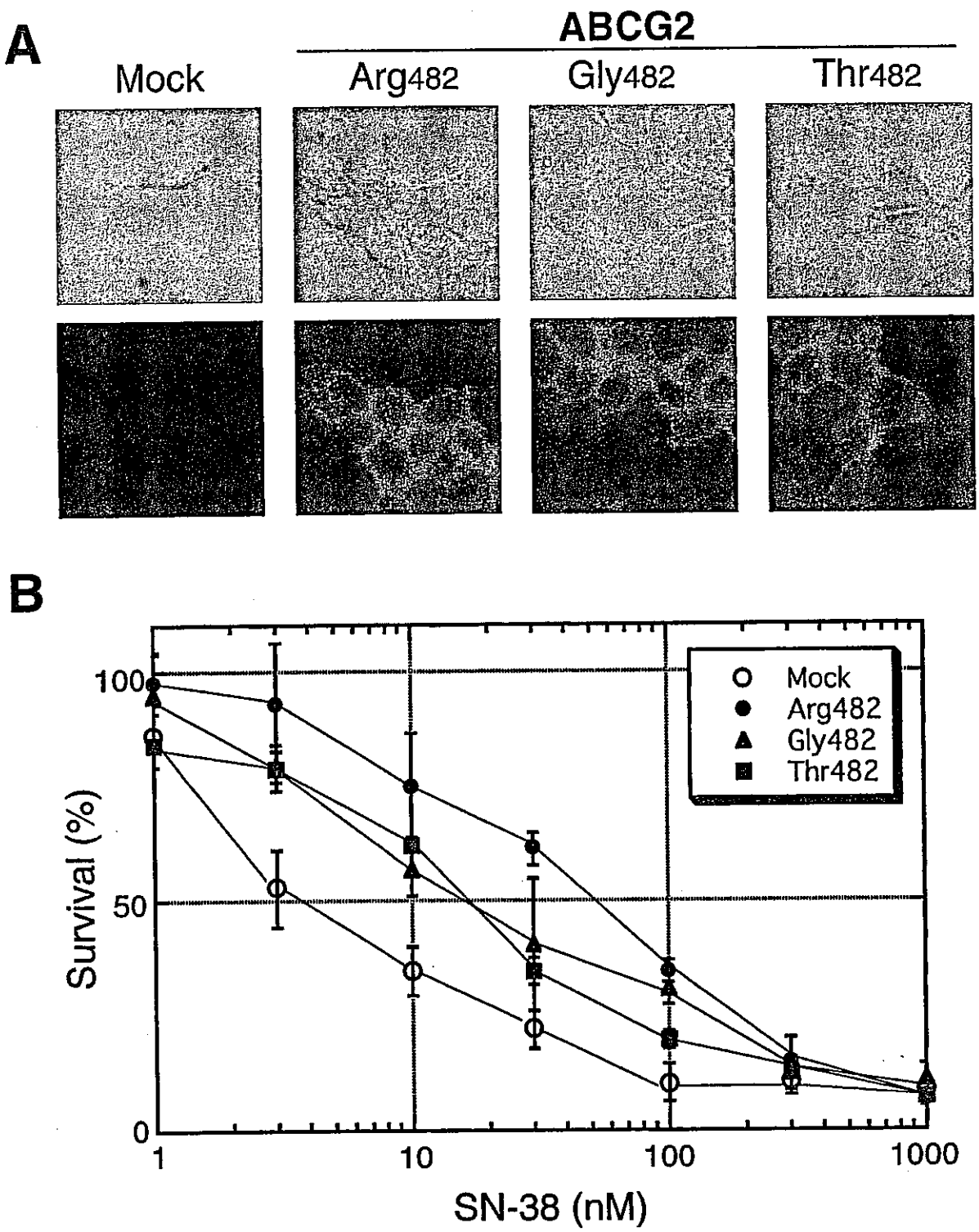

Figure 1. Expression of ABCG2 variant forms in HEK293 cells and their effect on the cellular sensitivity toward SN-38. Differential interference and immuno-fluorescence images of mock- and ABCG2 variant-transfected HEK293 cells (**A**). The ABCG2 protein was immunologically detected as described in MATERIALS AND METHODS. Cellular sensitivity of ABCG2-expressing HEK293 cells to SN-38 (**B**). HEK293 cells transfected with the mock vector or ABCG2 (Arg482, Gly482, and Thr482)-expression vectors were incubated in 100 μl of the culture medium containing SN-38 at different concentrations in 96-well plates. After incubation in a humidified tissue-culture chamber (5% CO_2) at 37°C for 72 h, surviving cells were detected by the MTT assay (see MATERIALS AND METHODS). Data are expressed as mean values ± S.D. of triplicate experiments.

Table 1. Drug resistance profiles of HEK293 cells transfected with a mock vector or ABCG2 variant-expression vectors.

Drug	IC_{50} [nM] (Resistance ratio)			
	Mock	Arg482	Gly482	Thr482
SN-38	3.1 ± 0.3	45.0 ± 3.1 (14.8)*	15.1 ± 2.1 (4.9)*	16.2 ± 2.2 (5.2)*
Mitoxantrone	1.7 ± 0.1	24.7 ± 0.1 (14.5)*	79.2 ± 1.0 (46.6)*	91.6 ± 2.9 (53.9)*
Doxorubicin	7.0 ± 0.6	8.5 ± 1.0 (1.2)	48.2 ± 4.5 (6.9)*	345 ± 25 (49.3)*
Daunorubicin	9.5 ± 1.2	11.0 ± 0.9 (1.2)	60.5 ± 4.8 (6.3)*	305 ± 23 (32.1)*
Vincristine	1.4 ± 0.1	1.4 ± 0.2 (1.0)	3.7 ± 0.3 (2.6)	9.0 ± 0.4 (6.4)*
Taxol	4.1 ± 0.2	8.0 ± 0.6 (2.0)	9.2 ± 0.5 (2.2)	16.0 ± 0.9 (3.9)
Etoposide	241 ± 22	270 ± 16 (1.1)	203 ± 20 (0.8)	233 ± 29 (1.0)
Mitomycin C	30.2 ± 2.1	28.3 ± 3.0 (0.9)	35.1 ± 2.2 (1.2)	45.3 ± 3.5 (1.5)
Methotrexate	58.1 ± 5.0	73.1 ± 6.2 (1.3)	62.0 ± 3.5 (1.1)	53.3 ± 4.5 (0.9)
Cisplatin	1103 ± 98	1051 ± 89 (1.0)	1201 ± 67 (1.1)	1104 ± 103 (1.0)

Cells were incubated with the drugs indicated in the table at different concentrations (1 nM to 10 μM). IC_{50} values were calculated as described in MATERIALS AND METHODS and are expressed as mean values ± S.D. (n = 3). Resistance ratio = (IC_{50} for ABCG2 variant-transfected cells)/(IC_{50} for mock vector-transfected cells).

**P<0.01 (Student's t-test)*

Profiling of drug resistance

A growth inhibition (IC_{50}) assay was performed by seeding HEK293 cells at a density of 2,000 cells per well in 96-well plates containing the culture medium (100 μl/well). After 24 h, drugs were added to the culture medium at different concentrations (1 nM to 10 μM), and cells were further incubated with the drug in a humidified tissue-culture chamber (37°C, 5% CO_2) for 72 h. To investigate the effect of natural flavonoids, cells were incubated in the presence of a flavonoid (20 μM) in the same manner. Surviving cells were detected by the MTT assay, as previously described (18). IC_{50} values were calculated from dose-response curves (i.e., cell survival vs. drug concentration) obtained in triplicate experiments.

Preparation of plasma membrane vesicles from HEK293 cells

HEK293 cells (approximately 2 x 10^9 cells) were harvested by centrifugation and suspended in 100 ml of ice-cold PBS. After centrifugation at 500 x *g* for 5 min, the cell pellet was diluted 40-fold with a hypotonic buffer (0.5 mM sodium phosphate pH 7.0 and 0.1 mM EGTA) and homogenized with a Potter-Elvehjem homogenizer. After centrifugation at 9,100 x *g*, the resulting supernatant was centrifuged at 100,000 x *g* for 30 min, and the resulting pellet was suspended in 250 mM sucrose containing 10 mM Hepes/Tris (pH 7.4). The crude membrane fraction was layered over a 38% (w/v) sucrose solution and centrifuged at 100,000 x *g* for 30 min. The turbid layer at the interface was collected, suspended in 250 mM sucrose containing 10 mM Hepes/Tris (pH 7.4), and centrifuged at 100,000 x *g* for 30 min. The membrane fraction was collected and resuspended in a small volume (150 to 250 μl) of 250 mM sucrose containing 10 mM Hepes/Tris (pH 7.4). After the protein concentration was measured by the BCA Protein Assay Kit (Pierce, Rockford, IL), the membrane suspension was stored at –80 °C until used.

Detection of ATP-dependent transport of SN-38 and its glucuronide conjugate

Frozen stocked membrane suspension was thawed quickly at 37°C, and vesicles were formed by passing the suspension through a 27-gauge needle. The standard incubation medium contained plasma membrane vesicles (50 μg of protein), 250 mM sucrose, 10 mM Hepes/Tris (pH 7.4), 10 mM $MgCl_2$, 5 mM ATP, 10 mM creatine phosphate, and 100 mg/ml of creatine kinase in a final volume of 80 μl. To examine the inhibitory effect of flavonoids, the standard incubation medium contained a flavonoid to be tested at various final concentrations (0.25 to 25 μM). The reaction was started by adding the substrate, i.e., SN-38 or SN-38glu, to the incubation medium. The reaction was carried out at 37°C, and the transport reaction was stopped at specified time points by the addition of 800 μl of ice-cold stop buffer solution containing 150 mM NaCl, 2 mM EDTA, and 10 mM Hepes/Tris (pH 7.4). The mixture was subsequently centrifuged at 16,000 x *g* for 30 min at 4°C, and the resulting precipitate was washed three times with the stop buffer solution (1.5 ml for each). The amount of the

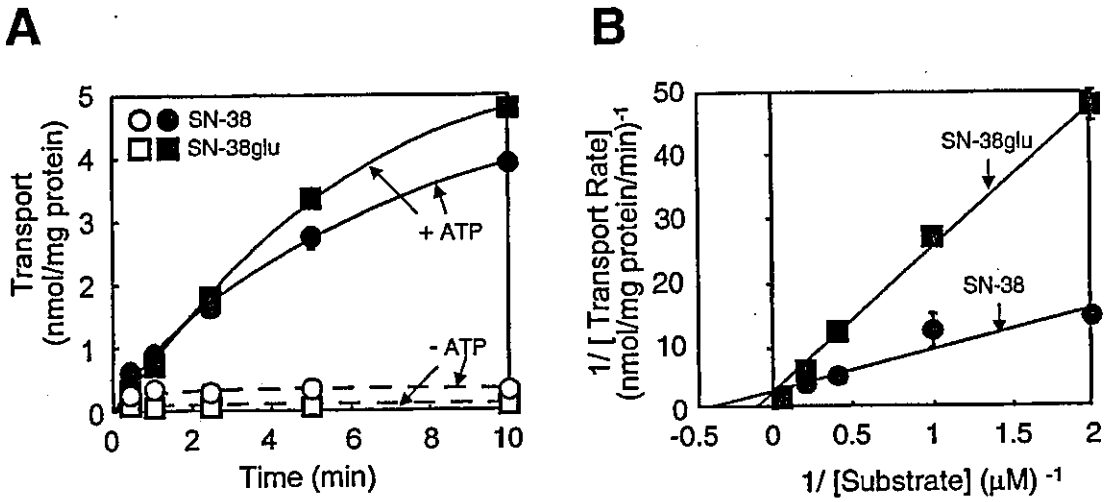

Figure 2. ATP-dependent transport of SN-38 and its glucuronide conjugate (SN-38glu) by [Arg482]ABCG2. Time courses of the transports of SN-38 carboxylate and SN-38glu in plasma membrane vesicles prepared from [Arg482]ABCG2-expressing HEK293 cells (**A**). The amount of SN-38 carboxylate (circles) or SN-38glu (squares) transported into plasma membrane vesicles was measured as described in MATERIALS AND METHODS. Lineweaver-Burk plots of ATP-dependent transport of the SN-38 carboxylate and SN-38glu (**B**). Substrate: SN-38 (closed circles) or SN-38glu (closed squares).

substrate incorporated into the vesicles was measured by the high-performance liquid chromatography (HPLC) method as described previously (12, 21). Kinetic parameters, such as the Km and Vmax values, for ATP-dependent transport of SN-38 or SN-38glu were calculated from Lineweaver-Burk plots. The Ki value for the inhibition of SN-38 transport by quercetin was determined from the data of Dixon plots.

RESULTS AND DISCUSSION

Stable expression of [Arg482], [Gly482], and [Thr482]ABCG2 in HEK293 cells and cellular resistance to SN-38

We have established ABCG2 variant-expressing HEK293 cells by transfection with the pcDNA3.1 vector carrying the cDNA of [Arg482], [Gly482], or [Thr482]ABCG2. Figure 1A demonstrates the differential interference (upper panels) and immuno-fluorescence images (lower panels) of those ABCG2 variant-expressing cells. Strong immuno-fluorescence was observed at the plasma membrane of ABCG2 cDNA-transfected cells, whereas no immuno-fluorescence was detected in mock vector-transfected HEK293 cells (negative control). These results provide evidence that three forms of human [Arg482], [Gly482], and [Thr482]ABCG2 variants were expressed predominantly in the plasma membrane of HEK293 cells.

Figure 1B shows the cellular resistance profiles of those ABCG2 variant-expressing HEK293 cells to SN-38. In this experiment, HEK293 cells were incubated with SN-38 (lactone form) at different concentrations (1 nM to 1 μM) at 37°C for 72 h. According to the IC_{50} values obtained with those cells, overexpression of [Arg482], [Gly482], and [Thr482]ABCG2 was found to confer HEK293 cells resistance to SN-38 by 14.8-, 4.9-, and 5.2-fold, respectively, as compared with the mock-transfected HEK293 cells (Figure 1B).

Drug resistance profile of [Arg482], [Gly482], and [Thr482]ABCG2-transfected HEK293 cells

To gain more insight into the association of those ABCG2 variants with drug resistance, we have tested a total of ten different anticancer drugs, including SN-38, mitoxantrone, doxorubicin, daunorubicin, vincristine, taxol, etoposide, mitomycin C, methotrexate, and cisplatin. Table 1 summarizes the drug resistance profiles of [Arg482], [Gly482], and [Thr482]ABCG2-overexpressing HEK293 cells; all three cell lines showed greatly enhanced drug resistance to mitoxantrone. Overexpression of [Thr482]ABCG2 resulted in 49- and 32-fold increases in drug resistance to doxorubicin and daunorubicin, respectively; whereas overexpression of

Table 2. Kinetic parameters for ATP-dependent transport of SN-38 and SN-38glu.

Variant	SN-38			SN-38glu		
	Km	Vmax	Vmax/Km	Km	Vmax	Vmax/Km
	(μM)	(pmol/min/mg protein)		(μM)	(pmol/min/mg protein)	
Arg482	3.1	455	146.7	21.7	909	41.9
Gly482	6.0	35	5.8	39.6	50	1.3
Thr482	3.7	36	9.7	34.6	62	1.8

Plasma membrane vesicles were prepared from HEK293 cells that expressed such ABCG2 variant forms as Arg482, Gly482, and Thr482. ATP-dependent transport of SN-38 carboxylate or SN-38glu was measured as described in MATERIALS AND METHODS. Km and Vmax values were calculated from Lineweaver-Burk plots as shown in Figure 2B.

[Arg482]ABCG2 did not significantly enhance the cellular resistance to those anticancer drugs. With respect to vincristine and taxol resistance, only modest elevations were found in [Thr482] and [Gly482]ABCG2-overexpressing HEK293 cells. No significant increase was observed in the resistance to etoposide, mitomycin C, methotrexate, or cisplatin in [Arg482], [Gly482], and [Thr482]ABCG2-overexpressing HEK293 cells (Table 1). These results suggest that overexpression of [Arg482], [Gly482], and [Thr482]ABCG2 confer different spectra of drug resistance profiles.

ATP-dependent transport of SN-38 and SN-38glu by [Arg482]ABCG2

SN-38 has an α-hydroxy-δ-lactone ring that undergoes reversible hydrolysis at neutral pH to form its carboxylate form. In the living cell, SN-38 is also metabolized by UDP-glucuronyl transferase to form SN-38glu. In the present study, we have examined ATP-dependent transport of both the carboxylate form of SN-38 and SN-38glu by using plasma membrane vesicles prepared from [Arg482], [Gly482], and [Thr482]ABCG2-overexpressing HEK293 cells. SN-38 lactone was non-specifically bound to plasma membrane vesicles, it was not possible to precisely detect the transport of the lactone form in our measurement system.

Figure 2A demonstrates time courses for the transport of SN-38 carboxylate and SN-38glu into plasma membrane vesicles prepared from [Arg482]ABCG2-overexpressing HEK293 cells. In the absence of ATP, the transport of those compounds was very low; however, it was remarkably enhanced by the addition of 5 mM ATP. Figure 2B depicts the Lineweaver-Burk plot of the ATP-dependent transport of SN-38 carboxylate and SN-38glu into plasma membrane vesicles from [Arg482]ABCG2-overexpressing HEK293 cells, showing simple Michaelis-Menten-type kinetics for the transport. Km values were estimated to be 3.1 and 21.7 μM for SN-38 carboxylate and SN-38glu, respectively (Table 2). While the Km value for SN-38 carboxylate was smaller than that for SN-38glu, the Vmax value for SN-38glu was about two-fold greater than that for SN-38 carboxylate. Based on the calculated Vmax/Km values (Table 2), it is suggested that [Arg482]ABCG2 transports SN-38 carboxylate more efficiently than SN-38glu. ATP-dependent transport of SN-38 carboxylate and SN-38glu were detected with plasma membrane vesicles prepared from [Gly482] and [Thr482]ABCG2-overexpressing HEK293 cells. However, as compared with [Arg482]ABCG2, the contribution of [Gly482] and [Thr482]ABCG2 to the transport of SN-38 carboxylate and SN-38glu is considered to be minimal (Table 2).

Contribution of [Arg482]ABCG2 to drug resistance against SN-38

Recently our group (18) and Chen et al. (19) have reported that [Arg482]ABCG2 transports methotrexate in an ATP-dependent manner. In contrast, no transport activity was observed with [Gly482] or [Thr-482]ABCG2. It is suggested that Arg at position 482 has a critical role in determining the substrate specificity of ABCG2 (16-20). Based on our findings, we have hypothesized that [Arg482]ABCG2 preferentially transports organic anions. As clearly demonstrated in this study, [Arg482]ABCG2 transports SN-38 carboxylate and SN-38glu, both of which are organic anions. It is important to note that the Km value (3.1 μM, Table 2) for SN-38 carboxylate was three orders of magnitude smaller than the Km value (5.7 mM, ref. 18) for methotrexate. Thus, it is concluded that [Arg482]ABCG2 has a much higher affinity towards SN-38 carboxylate than methotrexate.

Kawabata et al. have reported that an SN-38-selected PC-6/SN2-5H human lung carcinoma cell line overexpresses ABCG2 with the reduced intracellular accumulation of both SN-38 and SN-38glu (11). Plasma membrane vesicles prepared from that cell line transported both SN-38 and SN-38glu in an ATP-dependent manner (12). Furthermore, we have recently

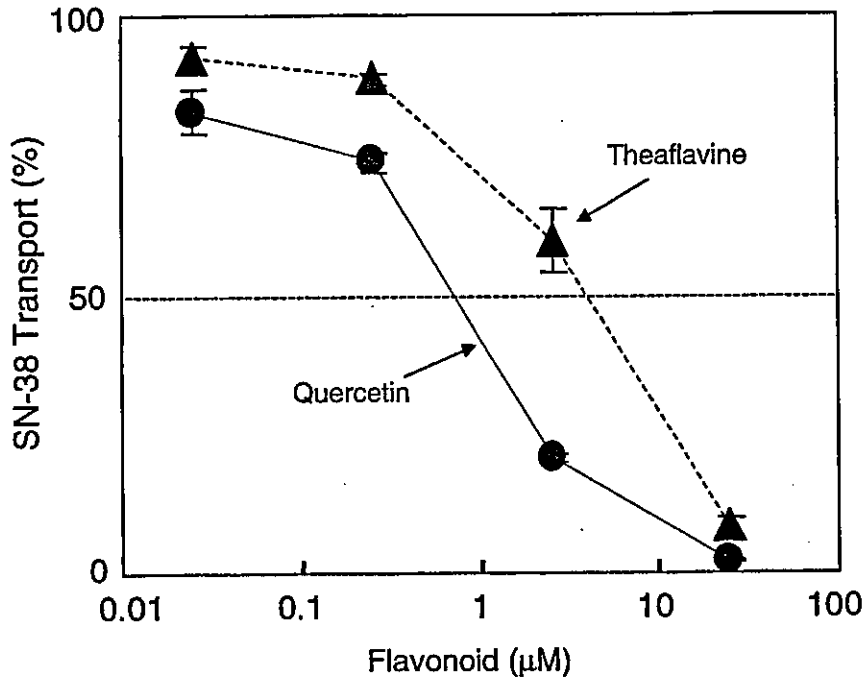

Figure 3. The effect of quercetin and theaflavine on the ATP-dependent transport of SN-38. Plasma membrane vesicles were prepared from [Arg482]ABCG2-expressing HEK293 cells and incubated with 2.5 μM SN-38 carboxylate in the presence of quercetin or theaflavine (0.025, 0.25, 2.5 or 25 μM). After the incubation at 37°C for 5 min, the amount of SN-38 transported into plasma membrane vesicles was measured as described in MATERIALS AND METHODS. Data are expressed as mean values ± S.D. of triplicate experiments.

cloned the ABCG2 cDNA from that cell line and confirmed that the amino acid at position 482 is Arg (Yoshikawa et al., manuscript submitted). Our present study provides more direct evidence for the ATP-dependent transport of SN-38 carboxylate and SN-38glu by exogenously expressed [Arg482]ABCG2 (Figure 2 and Table 2), and also demonstrates that overexpression of [Arg482]ABCG2 actually confers cells resistance to SN-38 (Table 1). When SN-38 lactone was incubated with PC-6 cells, the intracellular ratio of lactone/carboxylate was approximately 1:1 (21), suggesting a reversible conversion between the lactone and carboxylate forms of SN-38 within the cell. These observations are consistent with our findings. Taken together, it is strongly suggested that [Arg482]ABCG2 plays a major role in cellular resistance to SN-38 by actively extruding SN-38 carboxylate from cancer cells.

Inhibition of [Arg482]ABCG2-mediated SN-38 transport by flavonoids

To modulate the transport function of [Arg482] ABCG2 with natural compounds, we have tested flavonoids in our plasma membrane vesicle system. Table 3 shows the inhibitory effect of natural flavonoids

Table 3. *Effect of flavonoids on ATP-dependent transport of SN-38 in plasma membrane vesicles.*

Flavonoid	SN-38 transport	Inhibition
	(pmol/mg protein/min)	*(%)*
None	206.6 ± 1.1	0
Quercetin	4.4 ± 0.4	98
Theaflavine	17.2 ± 3.7	92
Theaflavine 3-O-gallate	102.9 ± 3.1	50
Epigallocatechin gallate	185.5 ± 8.3	10
Catechin	201.2 ± 0.9	3
Epigallocatechin	208.9 ± 2.8	0

Plasma membrane vesicles were prepared from [Arg482] ABCG2-expressing HEK293 cells and incubated with 2.5 μM SN-38 carboxylate in the absence or presence of 25 μM flavonoids at 37°C for 5 min. After the incubation, the amount of SN-38 transported into plasma membrane vesicles was measured as described in MATERIALS AND METHODS. Data are expressed as mean values ± S.D. of triplicate experiments.

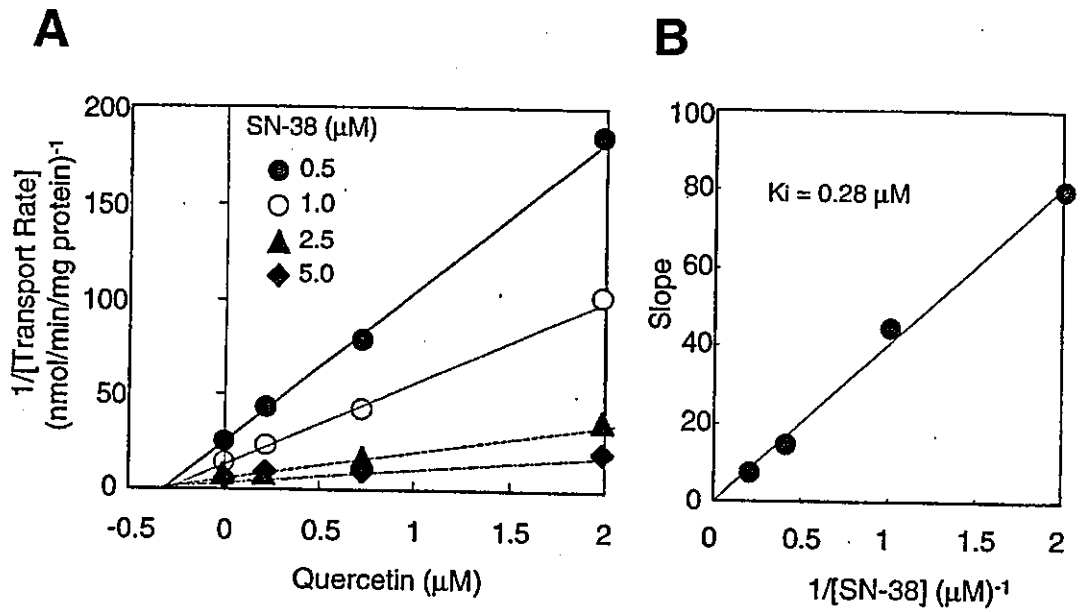

Figure 4. Inhibition of SN-38 transport by quercetin. Dixon plots of the rate of SN-38 transport vs. the reciprocal of quercetin concentration (**A**). The slope of the Dixon plots vs. the reciprocal of SN-38 concentration (**B**). Plasma membrane vesicles prepared from [Arg482]ABCG2-expressing HEK293 cells were incubated with SN-38 carboxylate (0.5, 1, 2.5 and 5 μM) in the presence of quercetin (0.2, 0.7, and 2 μM) at 37°C for 5min. The amount of SN-38 transported into plasma membrane vesicles was measured as described in MATERIALS AND METHODS. Data are expressed as mean values of triplicate experiments.

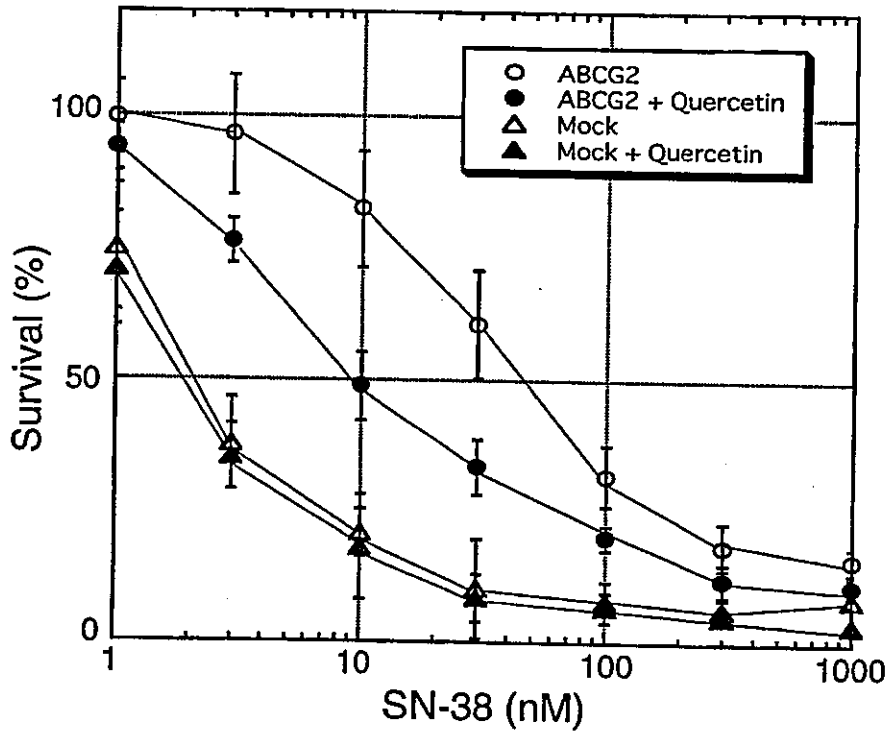

Figure 5. Effect of quercetin on the cellular resistance to SN-38. [Arg482]ABCG2-expressing and mock vector-transfected HEK293 cells were incubated in 100 μl of the culture medium containing SN-38 at different concentrations (0 to 100 nM) in the absence or presence of 20 μM quercetin. After incubation in a humidified tissue-culture chamber (5% CO_2) at 37°C for 72 h, surviving cells were detected by the MTT assay (see MATERIALS AND METHODS). Data are expressed as mean values ± S.D. of triplicate experiments.

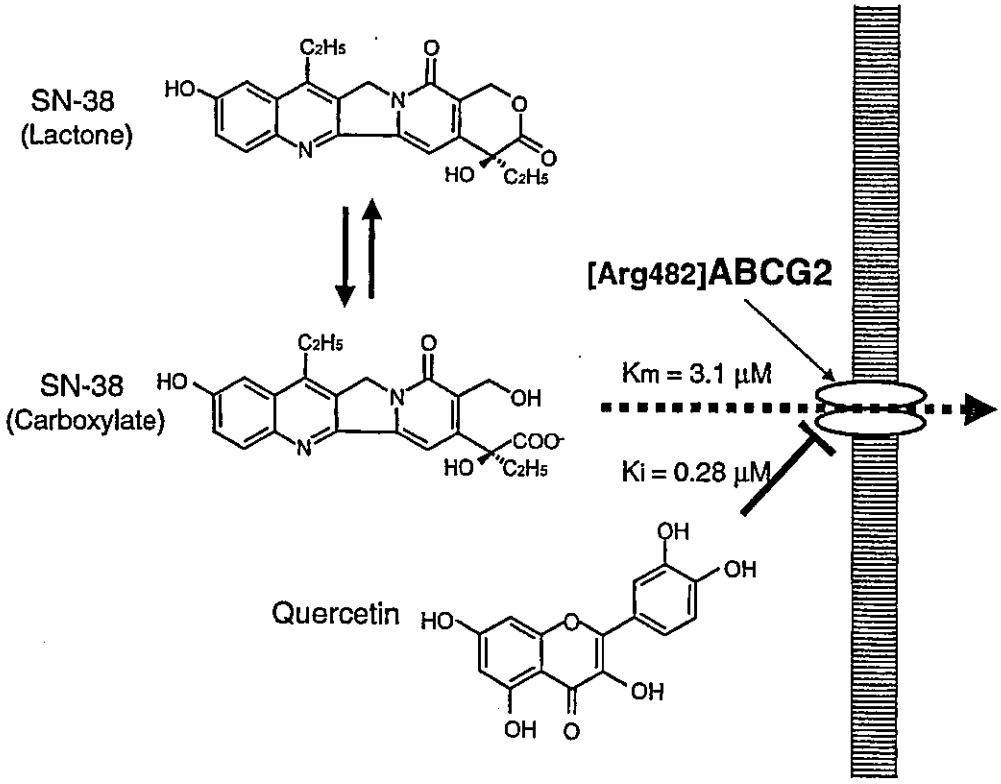

Figure 6. Schematic illustration for [Arg482]ABCG2-mediated transport of SN-38 carboxylate across the plasma membrane and its inhibition by quercetin. The α-hydroxy-δ-lactone ring of SN-38 undergoes reversible hydrolysis at neutral pH to form its carboxylate form (see ref. 20). SN-38 carboxylate is a good substrate for [Arg482]ABCG2 (Km = 3.1 μM) and thereby transported across the plasma membrane. On the other hand, the transport can be inhibited by quercetin (Ki = 0.28 μM).

(i.e., quercetin, theaflavine, theaflavine 3-*O*-gallate, epigallocatechin gallate, catechin, and epigallocatechin) on the ATP-dependent transport of SN-38 carboxylate. At the concentration of 25 μM, quercetin and theaflavine strongly (> 90%) inhibited SN-38 transport in plasma membrane vesicles prepared from [Arg482]ABCG2-transfected HEK293 cells. Catechin and epigallocatechin gallate had only small inhibitory effects, whereas epigallocatechin was without inhibitory effect. Figure 3 demonstrates that the inhibition by quercetin or theaflavine was dose-dependent; IC_{50} value was estimated to be 0.7 and 3.8 μM for quercetin and theaflavine, respectively. Quercetin was the strongest inhibitor among the flavonoids tested in this study. Based on Dixon plots (Figure 4), it has been revealed that quercetin competitively inhibited [Arg482]ABCG2, where the Ki value was estimated to be 0.28 μM. To our knowledge, this is the first report demonstrating the inhibition of ABCG2 by this natural flavonoid.

To determine whether quercetin could reverse ABCG2-associated drug resistance on the cellular level, we incubated [Arg482]ABCG2-transfected HEK293 cells with SN-38 at different concentrations in the presence of 20 μM quercetin. As shown in Figure 5, quercetin partly reversed the cellular resistance to SN-38. The IC_{50} value of [Arg482] ABCG2-transfected HEK293 cells has shifted from 45 nM to 10 nM in the presence of 20 μM quercetin (Figure 5). On the other hand, quercetin did not affect mock-transfected HEK293 cells at this concentration. Although quercetin strongly inhibited SN-38 transport in the plasma membrane vesicle system (Figures 3 and 4), its resistance-reversing effect in the cellular level was relatively weak (Figure 5). This difference may be due to low permeation of quercetin across the cell membrane and/or rapid metabolism of this flavonoid. Structural modifications of quercetin may improve the chemosensitizing potency in the cellular level.

Common features of SN-38 and quercetin molecules

The lactone E ring of SN-38 is reportedly an important pharmacophore for the inhibition of Topo I. Hydrolysis of the lactone E ring results in reduction of

anticancer activity of SN-38. As we recently reported, however, this lactone - carboxylate conversion reaction is reversible at neutral pH (ref. 21, see Figure 6 for schematic illustration). On the other hand, modifications of A or B rings do not significantly affect Topo I inhibition activity in vitro (22). In this context, we have recently synthesized CPT analogues with various substitutions at positions 10 or 11 of the A ring. At least one hydroxyl group attached to the A ring was found to be an important determinant for substrate recognition by [Arg482]ABCG2 (Yoshikawa et al. manuscript submitted). It is likely that hydrogen bond formation with such a hydroxyl group may be involved in substrate recognition or transport processes of ABCG2.

Interestingly, both SN-38 and quercetin have hydroxyl groups attached to the planar molecular structure (Figure 6). The planar structure with conjugated π-orbits may be critical for the interaction with the active site of the ABCG2 protein. Although catechin apparently resembles to quercetin, its molecular structure is not planar. This may explain the reason of why catechin and its metabolic derivatives (i.e. epigallocatechin, and epigallocatechin gallate) were weak inhibitors for ABCG2 (Table 3). Since [Arg482]ABCG2 exhibited a high affinity (Ki = 0.28 μM) toward quercetin, this natural product can be used as a molecular probe to characterize the active site of the ABCG2 protein.

CONCLUSION

Accumulating evidence suggests that drug resistance phenotypes vary among different cell lines expressing variant types of ABCG2 (16-20). In this regard, the present study provides direct evidence that [Arg482]ABCG2 does transport SN-38, the active metabolite of CPT-11, and thereby significantly contributes to cellular resistance to the anticancer drug. In addition, we have found that quercetin strongly inhibits the ATP-dependent SN-38 transport mediated by [Arg482]ABCG2. Since [Arg482]ABCG2 plays a key role in SN-38 resistance, quercetin or its derivatives are considered to be good candidates for the development of chemosensitizers. With this respect, it has recently been reported that combination of flavopiridol and CPT-11 augmented apoptosis and tumor regression in Hct116 colon cancer monolayers and xenografts (23). In addition, flavopiridol was also shown to prevent ABCG2-mediated mitoxantrone efflux in ABCG2-overexpressing cells (24).

The wild type (Arg482) of ABCG2 is expressed not only in cancer cells, but also normal cells. In particular, the apical localization in the epithelium of the small intestine and colon indicates a possible role of ABCG2 in the regulation of the uptake of p.o. administered drugs. In this context, natural flavonoids may serve as potent modulators for intestinal absorption of drugs as well as nutrients.

ACKNOWLEGEMENTS

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The genetic polymorphism of drug transporters: functional analysis approaches

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Keywords: ABC transporter, drug response, polymorphism, SNP, solute carrier family

Evidence is accumulating to strongly suggest that drug transporters are one of the determining factors governing the pharmacokinetic profile of drugs. To date, a variety of drug transporters have been cloned and classified as solute carriers and ATP-binding cassette transporters. Such drug transporters are expressed in various tissues such as the intestine, brain, liver, and kidney, and play critical roles in the absorption, distribution and excretion of drugs. However, at the present time, information is limited regarding the genetic polymorphism of drug transporters and its impact on their function. In this context, we have undertaken the functional analyses of the polymorphisms identified in drug transporter genes. This article aims to provide an overview on the functional aspects of the non-synonymous polymorphisms of drug transporters and to present standard methods for the evaluation of the effect of polymorphisms on their function.

Introduction

In the last decade of the 20th century, the development of high-throughput screening and combinatorial chemistry technologies accelerated the drug discovery process. In the 21st century, emerging genomic technologies (i.e., bioinformatics, functional genomics, and pharmacogenomics) are shifting the paradigm of drug discovery research and improving the strategy of medical care for patients [1]. Identifying human DNA sequences, genomic structures, and human genetic variations, along with changes in gene and protein expression, allows researchers and clinicians to more precisely define diseases and, in turn, to achieve the goal of 'personalized medicine'.

In order to realize personalized medicine, it is critically important to understand the molecular mechanisms underlying interindividual differences in the drug response, namely, pharmacological effect versus side effect. Varying drug responses among individuals may have a number of different causes, for example, genetic variations and/or expression levels of drug target molecules, including membrane receptors, nuclear receptors, signal transduction components, and enzymes, as well as those of drug-metabolizing enzymes and drug transporters. Observations of interindividual variations in different drug responses have led to the development of pharmacogenetics and pharmacogenomics [2,3].

Drug transporters and drug-metabolizing enzymes are important because they play pivotal roles in determining the pharmacokinetic profiles of drugs and, by extension, their overall pharmacological effects (i.e., drug absorption, drug distribution, drug metabolism and elimination, drug concentration at the target site, and the number and morphology of target receptors) [4-13]. The effects of drug transporters on the pharmacokinetic profile of a drug depend on their expression and functionality. Indeed, the expression of drug transporters can be modulated by endogenous and exogenous factors, including drugs themselves. It is also now known that inherited differences among individuals may also affect drug efficacy and toxicity [14-16]. Such inherited differences include genetic polymorphisms in drug targets and drug-metabolizing enzymes, as well as in drug transporters. To date, pharmacogenetics, the field dealing with such inherited differences and their effect on pharmacokinetics, has significantly contributed to our understanding of the genetic causes underlying differences in drug metabolism (e.g., cytochrome P450-mediated drug metabolism). In fact, recent technological advances allowing massive molecular sequencing have in turn allowed us to identify single nucleotide polymorphisms (SNPs) as one possible cause of variable drug response among individuals [17,18]. In light of such advances, it is important to carefully examine the clinical significance, if any, of polymorphisms in drug response genes, including drug transporters.

On September 6, 2000, the 43 members of the Japan Pharmaceutical Manufacturer's Association (JPMA) established the Pharma SNP Consortium (PSC) to conduct research into pharmacokinetic

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Table 1. ATP-binding cassette and solute carrier transporters expressed in small intestine, blood–brain barrier, liver and kidney.

Organ/tissue	ABC transporter	SLC transporter		
		Peptide transporter	Anion transporter	Cation transporter
Small intestine	ABCB1 (*P-gp/MDR1*) ABCB4 (*MDR2*) ABCC2 (*cMOAT/MRP2*) ABCC3 (*MRP3*) ABCC4 (*MRP5*) ABCC5 (*MRP5*) ABCC6 (*MRP6*) ABCG2 (*BCRP/MXR/ABCP*)	PEPT1 (*SLC15A1*)	MCT1 (*SLC16A1*) MCT4 (*SLC16A4*) MCT5 (*SLC16A5*) MCT8 (*SLC16A8*) OATP-B (*SLC21A9*) OATP-D (*SLC21A11*) OATP-E (*SLC21A12*) PGT (*SLC21A2*) AE2 (*SLC4A2*) ASBT (*SLC10A2*)	OCT1 (*SLC22A1*) OCTN1 (*SLC22A4*) OCTN2 (*SLC22A5*)
Blood–brain barrier	ABCB1 (*P-gp/MDR1*) ABCC1 (*MRP1*) ABCG2 (*BCRP/MXR/ABCP*)		MTC1 (*SLC16A1*) MCT2 (*SLC16A2*) OAT1 (*SLC22A6*) OAT3 (*SLC22A8*) OATP-A/OATP (*SLC21A3*)	OCTN2 (*SLC22A5*) OCT2 (*SLC22A2*) OCT3 (*SLC22A3*)
Liver	ABCB1 (*P-gp/MDR1*) ABCB4 (*MDR2*) ABCB11 (*SPGP/BSEP*) ABCC2 (*cMOAT/MRP2*) ABCC3 (*MRP3*) ABCG2 (*BCRP/MXR/ABCP*)		OATP-B (*SLC21A9*) OATP-C/LST-1 (*SLC21A6*) OATP-8 (*SLC21A8*) NPT1 (*SLC17A1*)	OCT1 (*SLC22A1*) OCTN2 (*SLC22A5*) NTCP (*SLC10A1*)
Kidney	ABCB1 (*P-gp/MDR1*) ABCC1 (*MRP1*) ABCC2 (*MRP2*)	PEPT1 (*SLC15A1*) PEPT2 (*SLC15A2*)	OAT1 ((*SLC22A6*) OAT3 (*SLC22A8*) OAT4 (*SLC22A11*) NPT1 (*SLC17A1*)	OCT1 (*SLC22A1*) OCT2 (*SLC22A2*) OCT3 (*SLC22A3*) OCTN1 (*SLC22A4*) OCTN2 (*SLC22A5*)

ABC: ATP-binding cassette; AE: Anion exchanger; ASBT: Apical sodium-dependent bile salt transporter; BCRP: Breast cancer-resistant protein; BSEP: Bile-salt export pump; cMOAT: Canalicular multispecific organic anion transporter; LST: Liver-specific transporter; MCT: Monocarboxylate transporter; MDR: Multi-drug resistance; MRP: Multi-drug resistance protein; NPT: Na^+/phosphate cotransporter; NTCP: Na^+/taurocholate cotransporting polypeptide; OAT: Organic anion transporter; OATP: Organic anion transporting polypeptide; OCT: Organic cation transporter; OCTN: Organic cation transporter novel type; PEPT: Peptide transporter; P-gp: P-glycoprotein; PGT: Prostaglandin transporter; SLC: Solute carrier; SPGP: Sister of P-glycoprotein.

gene polymorphisms in the Japanese population. During the period of 2000–2002, using blood samples donated by ~ 1000 Japanese volunteers, the PSC identified SNPs in ~ 180 pharmacokinetic genes, including drug-metabolizing enzymes and drug transporters. The PSC project then created a database of SNPs and information from expression and functional analyses of protein variants. The overall objective is to gather common fundamental data necessary for research into drug responsiveness in the Japanese population. In collaboration with the PSC, the authors have undertaken the functional analyses of SNPs discovered in drug transporter genes. This review is aimed at providing an overview on the polymorphisms of drug transporters and to provide standard methods for the evaluation of the effect of SNPs and mutations on their function.

Drug transporters: solute carriers and ATP-binding cassette transporter families

There is accumulating evidence to strongly suggest that drug transporters are one of the determinant factors governing the pharmacokinetic profile of drugs. Indeed, drug transporters are expressed in various tissues, such as the intestine, brain, liver, and kidney, to play critical roles in the absorption, distribution and excretion of drugs. To date, a variety of drug transporters have been cloned, and remarkable progress has been made in characterizing the molecular properties and functions of these transporters. Table 1 summarizes major drug transporters expressed in the small intestine, blood–brain barrier, liver, and kidneys. Those transporters have been classified as either primary or secondary active transporters. The primary active transporters include

Table 2. Substrate specificities and driving forces of SLC transporters.

Transporter	Substrate	Driving force
PEPT1, 2 (*SLC15A1, 2*)	Di- and tri-peptides, β-lactam antibiotics	Peptide/H^+ symport
OCTN1 (*SLC22A4*)	TEA, carnitine, quinidine, velapamil	Cation/H^+ exchange
OCTN2 (*SLC22A5*)	TEA, carnitine, acetylcarnitine	Na^+-dependent (carnitine, acetylcarnitine) Na^+-independent (TEA)
NPT1 (*SLC17A1*)	PAH, benzylpenicillin	Anion/Cl^- exchange (?)
OCTs (*SLC22A*)	TEA, MPP^+	Membrane potential-dependent
OATs (*SLC22A*)	PAH, β-lactam antibiotics, glutarate, cAMP, cGMP, urate, methotrexate, prostaglandin E_2	Anion/di-carboxylate exchange
OATP (*SLC21A*)	Bile salts (taurocholate, cholate)	Na^+-independent
NTCP (*SLC10A1*)	Taurocholate	Bile salt/Na^+ symport
MCT1 (*SLC16A1*)	Monocarboxylate, benzylpenicillin	Monocarboxylate/H^+ symport
AE2 (*SLCA2*)	Monocarboxylate	Anion/H^+ exchange

AE: Anion exchanger; MCT: Monocarboxylate transporter; MPP^+: 1-Methyl-4-phenylpyridinium; NPT: Na^+/phosphate cotransporter; NTCP: Na^+/taurocholate cotransporting polypeptide; OAT: Organic anion transporter; OATP: Organic anion transporting polypeptide; OCT: Organic cation transporter; OCTN: Organic cation transporter novel type; PAH: p-Aminohippurate; PEPT: Peptide transporter; SLC: Solute carrier; TEA: Tetraethylammonium.

ATP-binding cassette (ABC) transporters that utilize the ATP hydrolysis as the driving force for solute transport. On the other hand, the secondary transporters, for example, many solute carrier (SLC) transporters, are driven by an exchange of intra/extracellular ions [20] (Table 2). Each gene family of transporters is comprised of a multiplicity of members. The human Gene Nomenclature Committee has classified transporters by standardized names such as the SLC family and the ABC transporters [201].

The functions and substrate specificities of drug transporters have been characterized by several *in vitro* and *in vivo* techniques using cells expressing the transporter gene or using gene-knockout animals. In particular, construction of *in vitro* expression systems using human transporter cDNA clones provides useful models to evaluate substrate specificity. In addition, tissue distribution and levels of expression of the drug transporters convey important information for the prediction of the *in vivo* pharmacokinetic profile of drugs.

There are many factors that can affect the function as well as the expression of drug transporters. These factors may involve genetic mutations, SNPs, splicing, transcriptional regulation, stability of mRNA, post-translational modification, and intracellular localization. Evaluation of such factors is critically important in order to understand the whole picture of the pharmacogenomics of drug transporters. Functional analysis of drug transporter polymorphisms is one such important approach.

Solute carrier transporter genes

During the past decade, a large number of SLC transporter genes have been identified. It has been suggested that some of these transporters are responsible for drug transport in various tissues. For example, human peptide transporter 1 (PEPT1 [*SLC15A1*]) is an oligopeptide transporter expressed in the apical plasma membranes of intestinal and renal proximal epithelial cells. The protein consists of 708 amino acids and has 12 predicted transmembrane domain [21]. PEPT1 mediates intestinal (re)absorption of various drugs including β-lactam antibiotics, angiotensin-converting enzyme (ACE) inhibitors, the anticancer agent bestatine, and the antiviral agent valacyclovir, as well as di- and tripeptides from dietary nutrients [4]. The human novel organic cation transporter 1 (OCTN1 [*SLC22A4*]) gene encodes a 551 amino acid protein with 11 putative transmembrane domains and is expressed in several tissues including the kidney, bone marrow, trachea, skeletal muscle, and fetal but not adult liver [22]. Known

Table 3. Non-synonymous and synonymous polymorphisms in PEPT1, OCTN1, OATP-B, and OATP-C genes.

SNP name	NCBI SNP ID	JSNP ID	Nucleotide change	Codon change
PEPT1 reference sequence: NM_005073				
Sequence used as the standard: U21936.1				
PEPT1 (S117N)	rs2297322	ssj0008458	g406a	agc → aac
PEPT1 (Y167Y)	rs3737087	JST082067	c557t	tac → tat
PEPT1 (G419A)	rs4646227	ssj0005298	g1312c	ggc → gcc
PEPT1 (A449A)	rs1339067	ssj0008468	t1403c	gct → gcc
PEPT1 (V450I)	rs2274828	ssj0008469	g1404a	gta → ata
PEPT1 (R459C)	rs2274827	n/a	c1431t	cgc → tgc
OCTN1 reference sequence: NM_003059.2				
Sequence used as the standard: AB007448 (rs272893)				
OCTN1 (T306I)	NM_003059.2	ssj0005239	c1063t	aca → ata
OCTN1 (G462E)	rs4646201		g1531a	ggg → gag
OATP-B reference sequence: NM_007256				
Sequence used as the standard *1: AB026256				
OATP-B*2 (T392I)	rs1621378	n/a	c1175t	acc → atc
OATP-B*3 (S486F)	rs2306168	JST063697	c1457t	tct → ttt
OATP-C reference sequence: NM_006446				
Sequence used as the standard *1: AB026257				
OATP-C*1b (N130D)	rs2306283	ssj0003168	a388g	aat → gat
OATP-C*5 (V174A)	rs4149056	ssj0003182	t521c	gtg → gcg

JSNP: Japanese Single Nucleotide Polymorphisms database; NCBI: National Center for Biotechnology Information; OATP: Organic anion-transporting polypeptide; OCTN: Organic cation transporter novel type; PEPT: Peptide transporter; SNP: Single nucleotide polymorphism.

substrates are organic cations such as tetraethylammonium (TEA), quinidine, pyrilamine, verapamil, and carnitine [23]. OCTN1 has been supposed to contribute to the tissue distribution as well as renal elimination of cationic drugs [24]. Organic anion transporting polypeptides (OATPs/oatp) mediate the transport of a wide spectrum of amphipatic organic solutes and have recently been reclassified as a growing gene superfamily [25]. These are expressed in various organs and the distribution profile is extremely varied among each family member [25,26].

Databases of transporter SNPs have been established for Japanese as well as other ethnic populations. Saito *et al.* [27] and Iida *et al.* [28] screened DNA of 48 unrelated Japanese individuals for SNPs in many drug transporter genes of the SLC family by direct sequencing of their entire genomic regions except for repetitive-sequence elements. Although they have identified a number of SNPs and insertion/deletion polymorphisms, which have not been previously reported, the correlation between genotypes and phenotypes remains to be determined for most transporters.

SNPs of drug transporters may have potential impact on the pharmacokinetics of drugs. Indeed, organic cation/carnitine transporter OCTN2 (*SLC22A5*) is the causative gene for inherited primary systemic carnitine deficiency [29,202], and a single mutated amino acid residue in mouse OCTN2 resulted in decreased tissue distribution and renal secretory clearance of TEA [30]. S467C mutation in OCTN2 is an SNP found in the Japanese population. It shows increased Michaelis–Menten constant (K_m) value for carnitine by 15-fold while that for TEA remains unchanged [31]. SNPs in human organic anion-transporting polypeptides (OATP-C (*SLC21A6*) and OATP-B (*SLC21A9*) have been analyzed [32,33]. Moreover, Nishizato *et al.* [34] have investigated the contribution of OATP-C SNPs to the pharmacokinetics of pravastatine.

Functional analysis of single nucleotide polymorphisms of PEPT1, OCTN1, OATP-B and OATP-C

This section addresses the functional aspect of SNPs in SLC transporters, specifically PEPT1, OCTN1, OATP-B and OATP-C. The SNP alleles used in this study are summarized in Table 3. SNPs might affect the apparent activity of the transporter through various mechanisms; change in intrinsic activity because of lowered affinity for

substrates (K_m) and/or change in the translocation ability (the maximal uptake rate [V_{max}]). V_{max} could be altered by changes in the protein expression level or impaired subcellular sorting of the protein to appropriate domains of the plasma membrane. Therefore, it is critically important to examine protein expression levels for each transporter in the membrane. In the present study, we have used immunocytochemical and immunoblot analyses as well as the functional analysis for transporters to examine the effect of SNPs.

Design of study and interpretation of single nucleotide polymorphisms

The standard strategy for analyzing transporter SNPs is illustrated in Figure 1. HEK293 cell line can widely be used as the host cell system to express transporter proteins, because this cell line has an excellent efficacy of transient transfection in the conventional method of calcium phosphate precipitation [22]. The transporter cDNA should be subcloned into the appropriate multicloning site of an expression vector, such as pcDNA3 (Invitrogen, San Diego, CA, USA), that has a constitutive active promoter. By transfecting the cells with SLC transporters, the transport activity (i.e., the time-dependent and dose-saturable uptake by the transfected cells) can be detected. To discriminate specific uptake from endogenous background uptake, the uptake by cells transfected with pcDNA3 vector alone (mock) should be examined in parallel. The uptake activity measured with a substrate at a tracer concentration is expressed as the cell:medium ratio, which is a value obtained by dividing the amount of uptake by the cell per total cellular protein by its concentration in the transport medium. The uptake clearance can be evaluated from the linear regression of the uptake (cell:medium ratio) of the initial periods. The use of the uptake clearance is convenient to compare the uptake activity measured using different substrate concentrations. For instance, in the case of OCTN1-transfected cells, the uptake of standard substrate [^{14}C]TEA was increased time-dependently, it was linear up to 5 min and then reached a steady-state, whereas the uptake by the cells transfected with pcDNA3 vector alone exhibited a negligible increase with time, indicating specific uptake of the transfected transporter [22].

Correlation between expressed transporter protein and activity

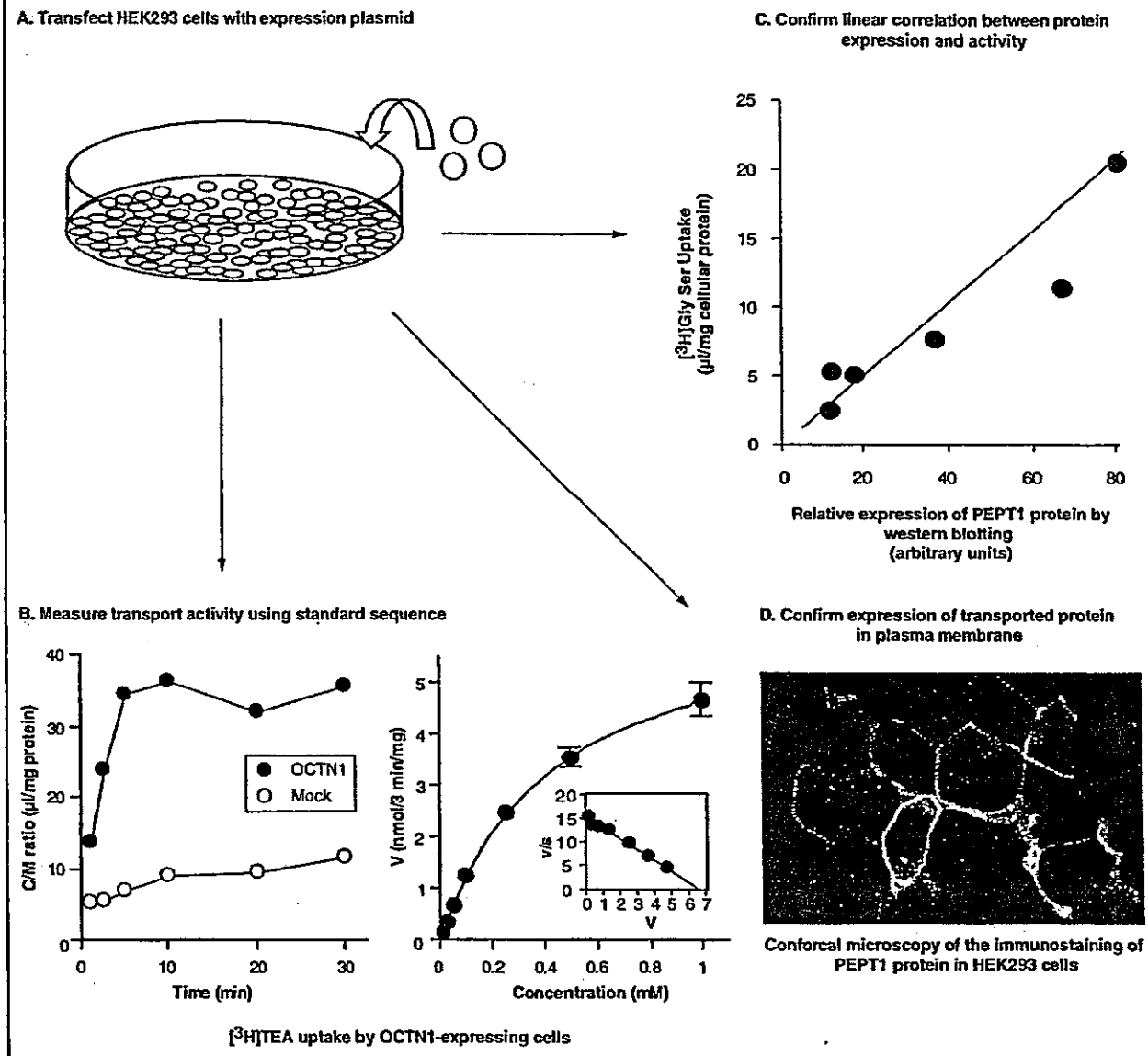
In order to evaluate the influence of SNP on the function of drug transporters, it is important to examine the expression levels of transporter proteins as well as their intrinsic activity. Changes in the expression level of the transporter can affect its V_{max} value. In the case of PEPT1, the relationship between the uptake activity of [^{3}H]glycylsarcosine and the protein expression level was evaluated (Figure 1C). HEK293 cells were transiently transfected with increasing amounts of the expression vector. The main band of PEPT1 was detected at a molecular mass of ~ 80 kDa in the transfected cells, whereas the band was absent in mock cells. As a linear relationship was obtained between the [^{3}H]glycylsarcosine uptake activity and the amount of expressed PEPT1 proteins (Figure 1C), it was confirmed that the transport activity of PEPT1 per the expressed protein is not saturated and that PEPT1 activity can be quantitatively evaluated.

Secondly, it is important to examine the subcellular localization of OATP-B by an immunocytochemical method. Figure 2 shows that OATP-B proteins expressed by transient transfection were predominantly localized in the plasma membrane [32], and no change was observed in the localization of OATP-B proteins among the three alleles. Accordingly, expression levels of OATP-B could be estimated by the densitometrical analysis of western blotting.

Kinetic analysis of transporter SNPs corrected for protein expression level

To evaluate the influences of SNPs on transport activity, three OATP-B alleles (OATP-B*1, OATP-B*2 and OATP-B*3) were expressed in HEK293 cells (Figure 2). All of the transfectants exhibited significantly greater [^{3}H]estrone-3-sulfate uptake activity compared with mock cells (data not shown). Therefore, transport activity of each allele was evaluated in terms of kinetic parameters K_m and V_{max} in the concentration range of 4 nM to 10 μM. The observed K_m values of [^{3}H]estrone-3-sulfate for OATP-B*1, OATP-B*2, and OATP-B*3 were 2.97, 2.32, and 2.31 μM, respectively, and the differences among these three SNPs were negligible. The V_{max} values of OATP-B*1, OATP-B*2, and OATP-B*3 were 332, 312, and 326 pmol/mg of total cellular protein/10 min, respectively, showing no apparent difference. However, these values should be corrected for the protein expression level of each transporter. To correct V_{max} per OATP-B protein, the expressed protein amount of each OATP-B SNP was quantitatively measured based

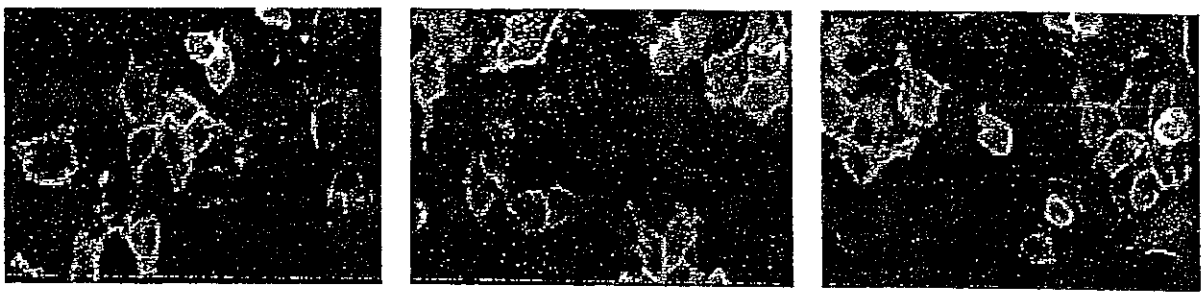
Figure 1. Strategy for functional analysis of solute carrier transporters expressed in HEK293 cells.



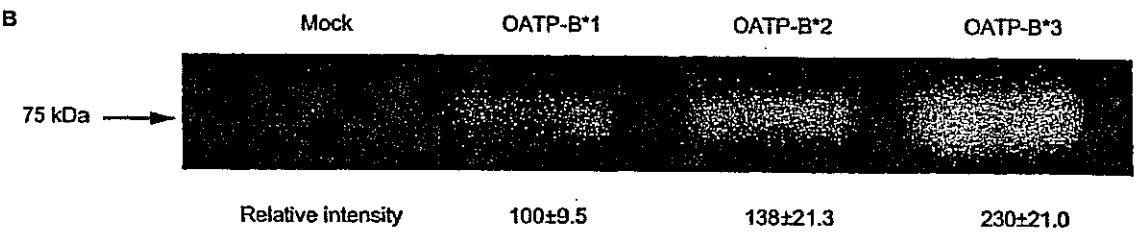
A. After 24 h cultivation of HEK293 cells in 15 cm dishes, the subconfluent cells were transfected with plasmid DNA by calcium phosphate precipitation method. At 48 h after transfection, the cells were harvested and suspended in the transport medium containing 125 mM NaCl, 4.8 mM KCl, 5.6 mM (+)-glucose, 1.2 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 25 mM HEPES at pH 7.4. **B.** Both the cell suspension and a solution containing a radiolabeled substrate in the transport medium (450 μl for each) were pre-incubated at 37°C for 20 min, and then the transport experiment was initiated by mixing them. At designated incubation times, aliquots (~ 200 μl) of the mixture were withdrawn and the cells were separated from the transport medium by centrifugal filtration through the oil layer. The associated radioactivity was measured by a liquid scintillation counter. **C.** HEK293 cells were transfected with different amounts of plasmid DNA (2–20 μg/dish). Western blot analysis of the crude membrane fractions was carried out and the immunoreactive protein was detected by using the ECL-plus western-blotting detection system (Amersham Biosciences). Quantitative analysis was made by densitometry using a Light Capture apparatus (Atto, Tokyo, Japan). Relative expression levels of the transporter protein were compared with the transport activity of the protein measured as described in B. **D.** HEK293 cells were grown on glass coverslips immersed in 15 cm dishes. Cells were then fixed and permeabilized with 3% formaldehyde in PBS for 1 min on ice, CH_3OH for 5 min on ice, and with 0.2% Triton X-100 in PBS. After the blocking, cells were incubated with the primary antibody and then with secondary antibody (Alexa Fluor 594 goat anti-rabbit IgG; Molecular Probes).
C/M: Cell:medium; OCTN: Organic cation transporter novel type; PEPT: Peptide transporter.

Figure 2. Estimation of kinetic parameters for OATP-B variants.

A



B



C

Substrate	Apparent kinetic parameters		Intrinsic activity
Estrone-3-sulfate	K_m (μm)	V_{max} (pmol/mg cell prot./10 min)	V_{max} (per OATP-B protein)
OATP-B*1	2.97±0.39	331.7±22.3	100
OATP-B*2	2.36±0.48	312.0±30.7	71.1
OATP-B*3	2.31±0.38	325.8±26.0	42.5

A. Localization of OATP-B*1, OATP-B*2, and OATP-B*3 proteins stably expressed in HEK293 cells. B. Western blot analysis of crude membrane fractions prepared from HEK293 cells transfected with vector alone (Mock) and the expression vector with OATP-B*1, OATP-B*2 or OATP-B*3 cDNA. Quantitative analysis was carried out as described in Figure 1B. C. Kinetic parameters (K_m and V_{max}) for the transport activity of wild type and allelic variants. These values were estimated by non-linear least-squares analysis for the concentration-dependent uptake of the substrates. Since the V_{max} value should depend on the expressed amount of transporter proteins, the obtained V_{max} was normalized with the results obtained by densitometry for western blot analysis. Data are expressed as mean values ± standard deviation (n = 3).
K_m: Michaelis–Menten constant; OATP: Organic anion-transporting polypeptide; V_{max}: Maximal uptake rate.

on the intensity of the western blot with the same batches of cells that were used for the evaluation of kinetic parameters for transport. The specific band for OATP-B was observed at ~ 75 kDa. The relative expression levels of OATP-B*1, OATP-B*2, and OATP-B*3 were 100, 138, and 230, respectively. Therefore the relative V_{max} values corrected with the expression level for OATP-B*1, OATP-B*2, and OATP-B*3 were 100, 71.1, and 42.5, respectively.

In conclusion, OATP-B*1 and OATP-B*2 gave no apparent difference in terms of protein expression levels, and subcellular localization, as well as intrinsic transport activity in terms of K_m and V_{max}. In the case of OATP-B*3, the intrinsic V_{max} value, which was corrected for protein expression level, was reduced by half, although neither the apparent activity nor subcellular localization remained unchanged. Therefore, the authors strongly advocate immunocytochemical analysis along with functional aspect analysis of SNPs in SLC tranpsorters. Detailed experimental data for OATP-C has been published by Nozawa *et al.* [32], and those for PEPT1 and