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Transport of dietary phenethyl isothiocyanate is mediated by multidrug resistance protein 2 but not P-glycoprotein

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Abstract

We demonstrated recently that phenethyl isothiocyanate (PEITC), a potent anticarcinogen present in cruciferous vegetables, inhibited P-glycoprotein (P-gp) and multidrug resistance protein 1 (MRP1) and that MRP1 can transport PEITC and/or its metabolites. In this study, we have examined whether PEITC is transported by P-gp and MRP2, two transporters with high expression in human intestine, liver and kidney. Using ^{14}C-PEITC, no significant difference was observed for the intracellular accumulation of PEITC in human breast cancer MCF-7/sensitive (control) and MCF-7/ADR (P-gp overexpressing) cells at PEITC concentrations of 1, 10 and 50 μM. Moreover, the presence of verapamil or PSC833, two P-gp inhibitors, had no significant effect on the intracellular accumulation of PEITC in P-gp overexpressing MCF-7/ADR and MDA435/LCC6MDR1 cells, indicating that PEITC may not be a substrate for P-gp. In contrast, ^{14}C-PEITC intracellular accumulation in the kidney epithelial MDCK II/MRP2 cells (transfected with human MRP2) was significantly lower than in the wild-type MDCK II/wt cells at PEITC concentrations of 1, 5, 10 and 50 μM. The presence of MK571, an MRP inhibitor, significantly enhanced ^{14}C-PEITC accumulation in MDCK II/MRP2 but not MDCK II/wt cells. Furthermore, depletion of intracellular glutathione (GSH) following treatment with buthionine sulphoximine, an inhibitor of GSH biosynthesis, significantly increased ^{14}C-PEITC intracellular accumulation in a concentration-dependent manner. Transcellular transport studies also demonstrated that depletion of intracellular GSH reduced the mean ratio of basal-to-apical transport to apical-to-basal transport of PEITC in MDCK II/MRP2, but not MDCK II/wt cell monolayers. These results indicate that GSH plays an important role in the MRP2-mediated transport of PEITC. The findings provide new information concerning the interactions between PEITC and membrane transporters and suggest the possibility of PEITC interactions with xenobiotics that are MRP2 substrates.

Keywords: Phenethyl isothiocyanate; P-glycoprotein; MRP2; Membrane transport

1. Introduction

P-glycoprotein (P-gp) and multidrug resistance proteins (MRPs) belong to the ATP binding cassette (ABC) superfamily of proteins and have been extensively studied. Active export of chemotherapeutic drugs by P-gp and MRP1 is believed to be important in multidrug resistance, which occurs when tumor cells possess intrinsic or acquired resistance to a broad spectrum of structurally and functionally diversified compounds. P-gp, a 170-kDa protein localized on the apical membrane of polarized cells, can extrude a wide range of structurally unrelated hydrophobic compounds, including important anticancer drugs such as anthracyclines, vinca alkaloids, epipodophylotoxins and taxanes [1,2]. P-gp is also expressed in many normal tissues, including the intestinal epithelium, blood brain barrier, hepatocytes and renal tubular cells, and plays an important role in drug absorption, elimination and distribution and protects the body against toxins [3,4].

MRP1, originally cloned from multidrug-resistant human lung cancer cells, is a 190-kDa protein localized to the basolateral membrane of epithelial cells [5]. MRP1 has a similar substrate spectrum of chemotherapeutic agents as reported for P-gp, but it also transports organic

Abbreviations: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; BSO, buthionine sulphoximine; GSH, glutathione; GST, glutathione-*S*-transferase; ITC, isothiocyanate; MRP, multidrug resistance protein; PEITC, phenethyl isothiocyanate; P_{app}, apparent permeability; P-gp, P-glycoprotein

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anions, such as drugs conjugated to glutathione (GSH), glucuronic acid or sulfate [6]. GSH plays an important role in MRP1-mediated efflux and free drugs may be transported in association with GSH [7]. MRP2 was originally referred to as the canalicular multispecific organic anion transporter (cMOAT). Although it shares 49% amino acid identity and similar substrate specificity with MRP1, MRP2 is different from MRP1 in several aspects. Unlike MRP1, but similar to P-gp, MRP2 is localized to the apical membranes of polarized cells. Compared with MRP1, which is ubiquitously expressed throughout the body, MRP2 has more limited tissue distribution with expression mainly in the liver, kidney and gut [6,8], and has much higher gene expression in human jejunum [9]. Animal studies showed that MRP2 is present in the brush border membrane surface of villi of the small intestine with the intensity decreasing from the villus tip to the crypts [10,11]. Consequently, MRP2 may limit the passage of certain xenobiotics or their metabolites from the intestinal tract into the circulation. MRP2 also has high expression on the bile canalicular membrane of heptocytes and has an important function in the biliary excretion of various types of anionic compounds including endogenous metabolites as well as many exogenous compounds. Human MRP2 gene deficiency, termed the Dubin–Johnson syndrome, results in a reduction in the biliary elimination of bilirubin glucuronides [12,13].

Organic isothiocyanates (ITCs, R–N=C=S), also known as mustard oils, are widely distributed in edible plants as their glucosinolate precursors. The Cruciferae family which includes many familiar vegetables (e.g. cabbage, broccoli, cauliflower, Brussels sprouts and kale) and >350 other genera that consist of many food plants (e.g. radish, daikon, watercress, horseradish and wasabi) are the richest sources of glucosinolates [14]. ITCs are released when the plants are damaged following chewing, cutting or grinding. The most striking biological activity of ITCs is their potent cancer chemopreventive effects in a broad spectrum of target tissues. In particular, phenethyl isothiocyanate (PEITC, Fig. 1) has pronounced antimutagenic activity against tobacco carcinogens since it is a potent inhibitor of their metabolic activation and the compound is being evaluated clinically as a chemopreventive agent for lung cancer [15,16]. The most important functional group responsible for the pharmacokinetics and pharmacodynamics of ITCs is the isothiocyanate group (–N=C=S). The central carbon atom on the isothiocyanate group is electrophilic and can easily react with O–, S–, and N–nucleophiles [17]. Binding of ITCs to the thiols present in proteins is believed to contribute to the many pharmacological and biological activities as well as the toxicities of

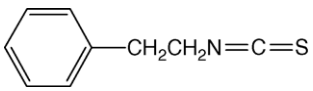

Fig. 1. Chemical structure of PEITC.

ITCs. Due to the abundant presence of GSH, the major thiol in cells, ITCs mainly accumulate within cells as GSH conjugates [18]. The conjugation can take place spontaneously, or is catalyzed by the enzyme glutathione-*S*-transferase (GST) [17]. It has been reported that GSH- and Cys-Gly-derivatives of PEITC accounted for 64.7% of the total derivatives released from the cells loaded with PEITC [19]. Although PEITC is a small hydrophobic compound that enters cells by passive diffusion, active transport of PEITC cannot be ruled out.

The human body is continuously exposed to exogenous chemicals present in the diet. Additionally, many of these dietary chemicals are ingested as herbal remedies and dietary supplements. As a consequence, the potential for pharmacokinetic interactions between drugs and phytochemicals, chemicals present in plants, becomes a concern [20]. Human daily intake of ITCs is estimated in the range of tens of milligrams [21] and daily intake of glucosinolates, the precursors of ITCs, has been reported to average 300 mg [22], although this amount can vary depending on the diet. For example, there is high consumption in Japan where daikon ingestion is estimated to be 20 kg/year or 55 g/day [22]. Indeed, understanding the potential interaction of PEITC with transporters is important since PEITC is a dietary compound regularly ingested by humans and is of extensive public and scientific interest as an anticarcinogenic and chemopreventive agent. Our laboratory has demonstrated that certain ITCs are inhibitors of the ABC transporters, P-gp and MRP1 [23,24]. In addition, PEITC and/or its cellular metabolites are substrates for MRP1 [24]. In the present study, we examined the accumulation and transport of PEITC mediated by P-gp and MRP2 in cells and evaluated the potential mechanisms underlying the interaction between PEITC and these ABC transporters.

2. Materials and methods

2.1. Materials

PEITC, verapamil and DL-buthionine (*R*, *S*)-sulphoximine (BSO) were purchased from Sigma–Aldrich (St. Louis, MO). MK571 was purchased from BIOMOL Research Labs (Plymouth Meeting, PA) and PSC833 was a kind gift from Novartis Pharma AG (Basel, Switzerland). ^{14}C-PEITC was synthesized in our laboratory with specific activity of 0.04994 mCi/mg and concentration of 1 mCi/ml. ^{3}H-mannitol (15 Ci/mmol) was purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA). RPMI 1640 medium, Dulbecco's Modification of Eagle's Medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), Hank's buffered salt solution (HBSS) and penicillin–streptomycin were supplied by Invitrogen (Buffalo, NY). The human breast cancer MCF-7/sensitive and MCF-7/ADR cell lines were obtained from National Cancer Institute. Human breast cancer MDA435/LCC6MDR1 cell was

kindly provided by Dr. Robert Clarke (Georgetown University, Washington, DC). The polarized Madin-Darby Canine Kidney MDCK II/wt (wild-type) and MDCK II/MRP2 (stably transfected with human MRP2) cells [25], were kind gifts from Dr. Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands).

2.2. Cell culture

Both sensitive and MDR subtypes of MCF-7 cells (used between passages 16 and 24) and MDA435/LCC6MDR1 cells (used between passages 16 and 28) were grown in RPMI 1640 culture medium containing 10% FBS. For MCF-7 cells, 50 IU/ml penicillin and 50 μg/ml streptomycin were supplemented. Both MCF-7 and MDA435/LCC6 are human breast cancer cell lines; MCF-7 is estrogen-dependent and MDA435/LCC6 is estrogen-independent. MDCK II/wt and MDCK II/MRP2 cells were incubated in DMEM culture medium supplemented with 10% FBS, 50 IU/ml penicillin and 50 μg/ml streptomycin. The cells were cultured in 75 cm^2 flasks in a humidified atmosphere with 5% CO_2/95% air at 37 °C.

2.3. GSH assay

MDCK II/wt and MDCK II/MRP2 cells grown in 35 mm^2 dishes were incubated with culture medium containing a specified concentration of BSO for 24 h to deplete intracellular GSH concentrations. BSO is an inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme of GSH biosynthesis [26]. Cells were then washed with ice-cold PBS three times, scraped into 5% sulfosalicylic acid (Sigma–Aldrich, St. Louis, MO), and lysed by sonication. Cell lysates were centrifuged at 10,000 × *g* for 10 min at 4 °C and the supernatant was measured for intracellular GSH concentrations using an enzymatic recycling procedure [27]. The enzyme kinetics were assessed by measuring the absorbance at 405 nm for 3 min at 30-s intervals and the sample GSH concentrations were determined from the calibration curve of δOD/min versus GSH standard concentration. GSH concentrations of the BSO-treated cells were presented as the relative values normalized by that of the non-treated cells.

2.4. ^{14}C-PEITC accumulation studies

^{14}C-PEITC accumulation studies were performed in MCF-7 (control and P-gp overexpressing) and MDA435/LCC6MDR1 (P-gp overexpressing) cells for studying P-gp-mediated transport and in MDCK II (wild-type and MRP2-expressing) cells for studying MRP2-mediated transport of PEITC. Briefly, cells grown in 35 mm^2 dishes were washed twice with sodium buffer containing 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 10 mM HEPES (pH 7.4) and then incubated with specified concentrations of ^{14}C-PEITC (supplemented with cold PEITC) in the presence or absence of inhibitors in sodium buffer for 1 h at room temperature. PSC833 (10 μM) and verapamil (100 μM), known inhibitors of P-gp, were used as positive controls in the P-gp study. MK571 (50 μM), a known inhibitor of MRP2, was used as a positive control in the MRP2 study. Cells were then washed three times with ice-cold stop buffer containing 137 mM NaCl, 14 mM Tris–base (pH 7.4) and lysed in 0.3 N NaOH-1% SDS. Aliquots of cell lysates were counted for cellular radioactivity by a liquid scintillation counter (1900 CA, Tri-Carb liquid scintillation analyzer, Packard Instruments Co., Meriden, CT) and measured for protein concentration by a BCA assay. Cellular accumulation was normalized by protein concentration and expressed as the percentage of the control group.

2.5. ^{14}C-PEITC transport studies

^{14}C-PEITC transcellular transport studies using MDCK cell monolayers were conducted as previously described [28]. Briefly, cells were seeded in Transwell polycarbonate inserts (6-well, 0.4-μm pore size, Corning Costar Corp.) at a density of 10^6 cells/well and grown for 7–9 days. Cells for GSH depletion studies were treated with 100 μM BSO for the last 24 h during their culture. The monolayers with TEER values higher than 1500 Ω cm^2 were used for the experiments. During the study, cells were washed twice with warm HBSS for 10 min each time and ^{14}C-PEITC (5 μM) was added to either the apical or basolateral chamber (donor chamber). Samples (100 μl) were taken from the opposite chamber (receiver chamber) at 7, 15, 30 and 60 min following the addition of ^{14}C-PEITC, and the same volume (100 μl) of HBSS buffer was immediately replaced in the receiver chamber. Samples were measured by a liquid scintillation counter. To ensure the integrity of the monolayer, a parallel experiment was conducted in triplicate to measure the apparent permeability coefficients (P_{app}) of ^{3}H-mannitol, a paracellular marker, across cell monolayers in both apical-to-basolateral (AP-to-BL) and basolateral-to-apical (BL-to-AP) directions. P_{app} across the cell monolayers in both the apical-to-basolateral ($P_{app, AP\text{-}to\text{-}BL}$) and basolateral-to-apical ($P_{app, BL\text{-}to\text{-}AP}$) directions were calculated using the following equation:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0}$$

where $\Delta Q/\Delta t$ represents the rate of ^{14}C-PEITC appearing in the receiver chamber, C_0 the initial concentration of ^{14}C-PEITC in the donor chamber, and A the surface area of the cell monolayer (4.7 cm^2).

2.6. Statistical analysis

Statistical analysis was conducted using a Student's *t*-test or a one-way ANOVA followed by Bonferroni's post hoc test. Results were considered statistically different when $P < 0.05$.

3. Results

3.1. ^{14}C-PEITC accumulation in parental and P-gp-overexpressing cells

Our laboratory demonstrated previously that PEITC is a P-gp inhibitor [23,24]. To examine whether PEITC is a P-gp substrate, the intracellular accumulation of ^{14}C-PEITC was investigated in parental (MCF-7/sensitive) and P-gp overexpressing (MCF-7/ADR and MDA435/LCC6 MDR1) cells in the presence and absence of known P-gp inhibitors. Western blot analysis showed that both MCF-7/ADR and MDA435/LCC6MDR1 cells have significant expression of P-gp whereas MCF-7/sensitive has no detectable expression of P-gp; none of the three cell lines has detectable expression of MRP1 [29]. As shown in Fig. 2A, the accumulation of PEITC was not significantly different in MCF-7/sensitive and MCF-7/ADR cells at PEITC concentrations of 1, 10 and 50 μM, indicating that there was no P-gp-mediated efflux of PEITC. Moreover, the presence of PSC833 or verapamil, two potent inhibitors of P-gp, had no affect on the intracellular accumulation of PEITC in either MCF-7/sensitive or MCF-7/ADR cells. Consistent with that observed in MCF-7/ADR cells, in estrogen-independent MDA435/LCC6MDR1 cells, another well established MDR human breast cancer cell line with higher expression of P-gp than MCF-7/ADR [29], the addition of 10 μM PSC833 had no effect on PEITC accumulation (Fig. 2B). Taken together, PEITC is not likely to be transported by P-gp; however, P-gp-mediated transport of PEITC cannot be excluded at PEITC concentrations lower than 1 μM.

3.2. ^{14}C-PEITC accumulation in MDCK II/wt and MDCK II/MRP2 cells

To examine the interaction between PEITC and MRP2, we studied the concentration-dependent accumulation of ^{14}C-PEITC in kidney epithelial MDCK II/wt and MDCK II/MRP2 cells [25]. Western blot analysis in our laboratory showed that MDCK II/MRP2 cells have pronounced expression of MRP2 whereas MDCK II/wt cells do not have detectable MRP2 expression (data not shown). At PEITC concentrations of 1, 5, 10 and 50 μM, the intracellular level of ^{14}C in MDCK II/MRP2 cells was substantially lower than the MDCKII/wt cells, being only 55, 14, 12 and 61 of that in MDCK II/wt cells, respectively ($P < 0.01$ for all the concentration groups) (Fig. 3). The export of PEITC at the lowest concentration, 1 μM, was less than expected, probably due to low specific activity of synthesized ^{14}C-PEITC and thus poor measurement accuracy. Nonetheless, at the other three concentrations, the extent of efflux generally decreased as PEITC concentration increased, showing a saturable pattern. Moreover, the addition of MK571 (50 μM), a potent inhibitor of MRP, dramatically enhanced intracellular ^{14}C-radioactivity

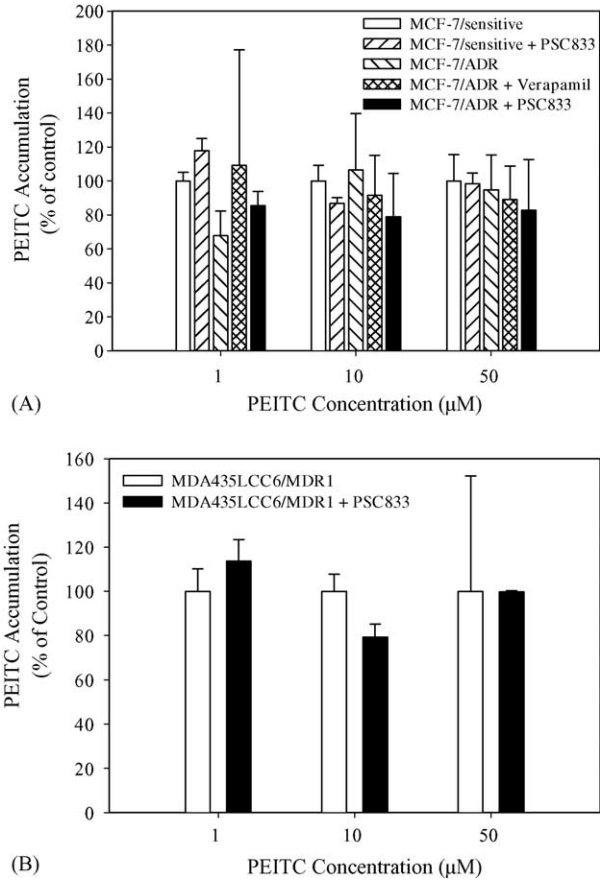

Fig. 2. PEITC accumulation in control and P-gp overexpressing cells. ^{14}C-PEITC accumulation was measured in MCF-7/sensitive (control) and MCF-7/ADR (P-gp overexpressing) cells (A) and MDA435LCC6/MDR1 (P-gp overexpressing) cells (B). Cells were incubated with specified concentrations of ^{14}C-PEITC (supplemented with cold PEITC) in the absence or presence of PSC833 (10 μM) or verapamil (100 μM) for 1 h. The intracellular PEITC accumulation was normalized by the cellular protein and presented as the percentage of the accumulation amount in MCF-7/sensitive cells compared with control (without inhibitor) (A) or the percentage of the accumulation amount in MDA435LCC6/MDR1 cells compared with control (without inhibitor) (B). Data are expressed as mean ± S.D., $n = 6$.

accumulation in MDCK II/MRP2 cells by 2.5-, 3.5-, 4.5- and 1.8-fold, respectively, in the presence of 1, 5, 10 and 50 μM ^{14}C-PEITC ($P < 0.01$ for all the concentration groups). Since the intracellular ^{14}C-radioactivity measured represent both PEITC and its cellular metabolites containing the radiolabel, these results indicate that PEITC and/or its cellular metabolites are transported by MRP2. In MDCKII/wt cells, MK571 had no or a small effect on intracellular PEITC accumulation (Fig. 3).

3.3. Effect of intracellular GSH on ^{14}C-PEITC accumulation in MDCK II/wt and MDCK II/MRP2 cells

GSH conjugates are often substrates for MRP and GSH is regarded as a co-substrate for MRP-mediated transport [7]. As it is known that the major cellular metabolite of PEITC is the GSH conjugate, we evaluated the effect of

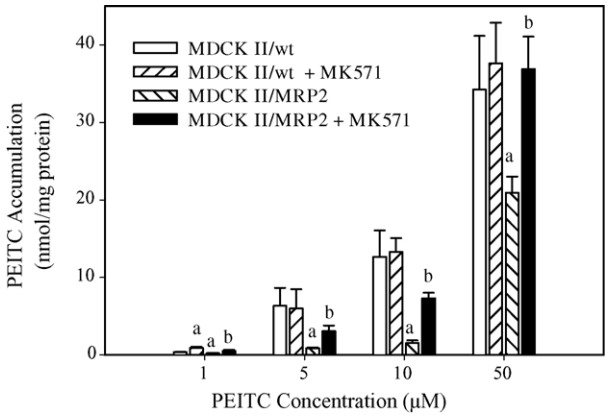

Fig. 3. Intracellular ^{14}C-PEITC accumulation in MDCK II/wt and MDCK II/MRP2 cells. Cells were incubated with specified concentrations of ^{14}C-PEITC (supplemented with cold PEITC) in the absence or presence of MK571 (50 μM) for 1 h. The intracellular PEITC accumulation was normalized by the amount of cellular protein and are expressed as mean ± S.D., $n = 6$. Statistical analysis was performed by a Student's t-test; [a] $P < 0.01$ compared to MDCK II/wt cells treated with the same concentration of PEITC in the absence of MK571; [b] $P < 0.01$ compared to MDCK II/MRP2 cells treated with the same concentration of PEITC in the absence of MK571.

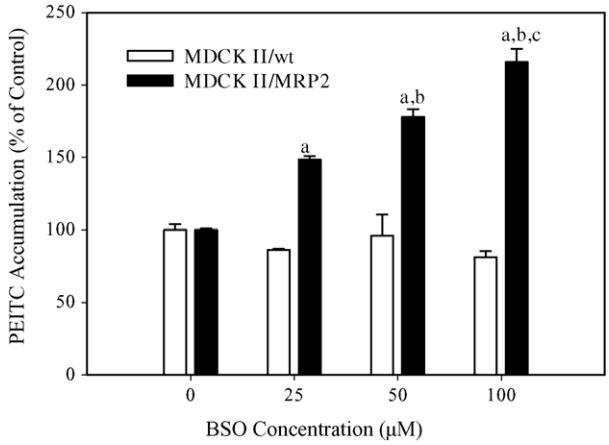

Fig. 4. Intracellular ^{14}C-PEITC accumulation in MDCK II/wt and MDCK II/MRP2 cells following BSO treatment. Following treatment by specified concentrations of BSO for 24 h, MDCK II/wt and MDCK II/MRP2 cells were incubated with 5 μM ^{14}C-PEITC for 1 h. Intracellular accumulation of ^{14}C-PEITC was normalized by cellular protein amount and expressed as percent of control cells that were not pretreated by BSO. Data are presented as mean ± S.D., $n = 6$. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post hoc test; [a] $P < 0.001$ compared to the corresponding control cells (without BSO treatment); [b] $P < 0.001$ compared to the corresponding cells treated with 25 μM of BSO; [c] $P < 0.001$ compared to the corresponding cells treated with 50 μM of BSO.

intracellular GSH on the accumulation of PEITC in MDCK II/wt and MDCK II/MRP2 cells to understand the role of GSH in the interaction between MRP2 and PEITC. After treatment with 25, 50 and 100 μM of BSO, the intracellular concentration of GSH was reduced in a concentration-dependent manner, being only approximately 46, 36 and 28% of the original cellular GSH level for both cell lines ($P < 0.001$ compared to the control) (Table 1). Consistent with the gradual decrease of intracellular GSH level resulting from treatment with 25, 50 and 100 μM of BSO, the intracellular accumulation of ^{14}C-radioactivity increased gradually from 100 ± 0.84% (control, no BSO treatment) to 148 ± 2.5, 177 ± 5.3 and 216 ± 9.1% after a 1 h-incubation of 5 μM ^{14}C-PEITC ($P < 0.001$ for all the groups) (Fig. 4). In addition, significant differences existed among cells treated with different concentrations of BSO in terms of both intracellular GSH level and intracellular PEITC accumulation, showing consistent dose-dependent effects (Table 1, Fig. 4). Therefore, the less GSH in MRP2-expressing cells, the more radiolabeled PEITC and/or metabolites remained inside cells, suggesting that GSH is critical in MRP2-mediated efflux of PEITC and/or its cellular metabolites. In MDCK II/wt cells, the depletion of intracellular GSH had no or a small effect on intracellular accumulation of PEITC.

Table 1
Intracellular GSH concentrations with and without BSO treatment

BSO concentration (μM)	Relative intracellular GSH level	
	MDCK II/wt cell	MDCK II/MRP2 cell
0 (control)	1.00 ± 0.05	1.00 ± 0.07
25	0.46 ± 0.03[***]	0.47 ± 0.05[***]
50	0.36 ± 0.08[***]	0.36 ± 0.04[***]
100	0.28 ± 0.03[***,b]	0.28 ± 0.004[***,c]

Cells were treated with BSO for 24 h and intracellular GSH concentrations were measured as described in Section 2. Cellular GSH concentrations are presented as the relative value of the corresponding control cells (without BSO treatment). Statistical analysis was conducted by one-way ANOVA followed by Bonferroni's post hoc test.

[***] $P < 0.001$ compared with control cells.

[b] $P < 0.05$ compared with cells treated with 25 μM BSO, $n = 3$.

[c] $P < 0.01$ compared with cells treated with 25 μM BSO, $n = 3$.

3.4. Effect of intracellular GSH on ^{14}C-PEITC transcellular transport across MDCK II/wt and MDCK II/MRP2 monolayers

To confirm the observations from the PEITC accumulation study in MDCK cells, we further examined the effect of intracellular GSH on the transcellular transport of ^{14}C-PEITC across the MDCK II/wt and MDCK II/MRP2 cell monolayers. It is known that MRP2 is localized to the apical membrane of MDCK II/MRP2 cells [25]. All the P_{app} values of ^{3}H-mannitol across MDCK II/wt and MDCK II/MRP2 were lower than 1.00×10^{-6} cm/s, indicating little paracellular transport; additionally, there was no significant difference between AP-to-BL and BL-to-AP transport of ^{3}H-mannitol (data not shown). Consistent with the observations in the accumulation study, the $P_{app,\ BL\text{-}to\text{-}AP}$ value of ^{14}C-PEITC across MDCK II/MRP2 cell monolayers ($8.24 \pm 0.52 \times 10^{-7}$ cm/s) was considerably higher than the $P_{app,\ AP\text{-}to\text{-}BL}$ value ($1.92 \pm 0.18 \times 10^{-7}$ cm/s, $P < 0.001$) with a mean transport ratio ($P_{app,\ BL\text{-}to\text{-}AP}/P_{app,\ AP\text{-}to\text{-}BL}$) of 4.3 (Fig. 5A), indicating that PEITC and/or its cellular metabolites are actively transported out of the

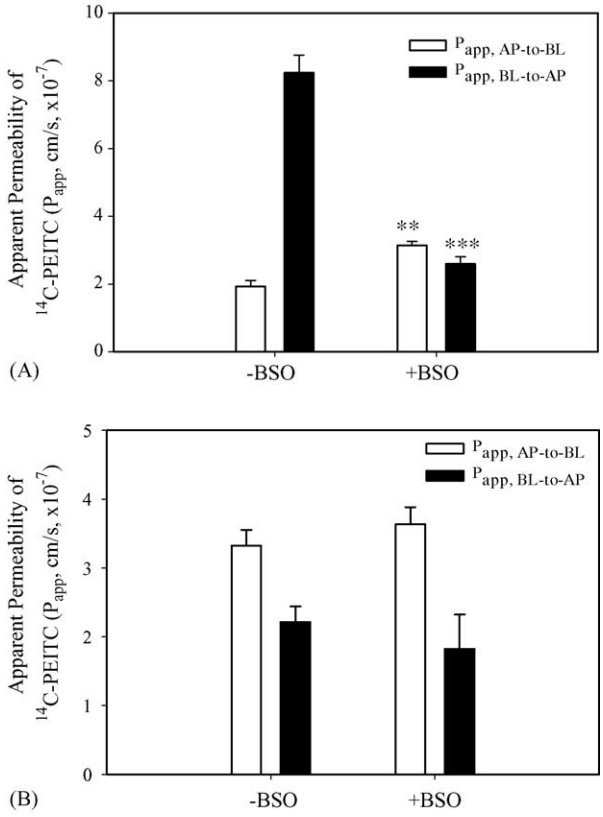

Fig. 5. Apparent permeability of PEITC across MDCK II/wt and MDCK II/MRP2 cell monolayers. MDCK II/wt and MDCK II/MRP2 cells were plated on transwells and incubated with or without BSO for a period of 24 h. ^{14}C-PEITC (5 μM) was added to the donor chamber and samples were taken from the receiver chamber after 7, 15, 30 and 60 min. The apparent permeability of PEITC (P_{app}) across MDCK II/MRP2 (5A) and MDCK II/wt (5B) cell monolayers were determined in both apical-to-basolateral ($P_{app,\ AP\text{-}to\text{-}BL}$) and basolateral-to-apical ($P_{app,\ BL\text{-}to\text{-}AP}$) directions. Data are expressed as mean ± S.D., $n = 3$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the corresponding control P_{app} (without BSO treatment) by a Student's t-test.

cells at the apical membrane. After treatment with 100 μM BSO for 24 h which caused intracellular GSH concentrations to decrease to 28% of the original level (Table 1), $P_{app,\ BL\text{-}to\text{-}AP}$ across MDCK II/MRP2 cells significantly decreased ($2.59 \pm 0.21 \times 10^{-7}$ cm/s, $P < 0.001$) whereas $P_{app,\ AP\text{-}to\text{-}BL}$ significantly increased ($3.13 \pm 0.12 \times 10^{-7}$ cm/s, $P < 0.01$) with a mean transport ratio of 0.83 (Fig. 5A), indicating that depleted GSH resulted in significantly reduced BL-to-AP transport and significantly enhanced AP-to-BL transport of ^{14}C-PEITC/metabolites. In MDCK II/wt cells, substantial abolishment of BL-to-AP directional transport was not observed and no significant difference was observed for either $P_{app,\ BL\text{-}to\text{-}AP}$ or $P_{app,\ AP\text{-}to\text{-}BL}$ value before and after treatment with 100 μM BSO (Fig. 5B). Like BSO-treated MDCK II/MRP2 cells, the $P_{app,\ AP\text{-}to\text{-}BL}$ of ^{14}C-PEITC/metabolites was higher than the $P_{app,BL\text{-}to\text{-}AP}$ across MDCK II/wt cell monolayers, with mean transport ratios of 0.67 and 0.50 without and with 100 μM BSO treatment, respectively. These ratios (less than 1) could be due to the expression of other endogenous efflux transporters on the basolateral membrane or uptake transporters on the apical membrane of the cells. Nevertheless, both accumulation and transcellular transport studies demonstrated that intracellular GSH is important in the MRP2-mediated transport of PEITC.

4. Discussion

Our laboratory has demonstrated that PEITC can inhibit ABC transporters including P-gp, MRP1 and breast cancer resistance protein (BCRP) [23,24,30]. In the current study, we found that PEITC and its cellular metabolites do not represent substrates of P-gp when evaluated using two different types of cell lines, MCF-7 and MDA435/LCC6. Of the two P-gp-overexpressing cell lines, MDA435/LCC6MDR1 has higher P-gp expression than MCF-7/ADR cells [29]. The intracellular accumulation of ^{14}C-PEITC was not significantly different in the P-gp overexpressing and the parental MCF-7 cells. Moreover, the presence of P-gp inhibitors, PSC833 or verapamil, had no effect in ^{14}C-PEITC accumulation in P-gp overexpressing MCF-7/ADR or MDA435/LCC6MDR1 cells. However, at ^{14}C-PEITC incubation concentration of 1 μM, although not statistically significant, accumulation of ^{14}C-PEITC was lower in MCF-7/ADR cells than in MCF-7/sensitive cells and a small increase of ^{14}C-PEITC accumulation was observed in the presence of a P-gp inhibitor in MCF-7/ADR and MDA435/LCC6MDR1 cells. Due to the low specific activity of radiolabelled PEITC and the sensitivity limitation of liquid scintillation counting, incubating cells with 1 μM ^{14}C-PEITC resulted in low measurement values and high variability. Consequently, we were not able to study PEITC concentrations lower than 1 μM. Therefore, we conclude that PEITC and its metabolites do not represent good substrates for P-gp. However, P-gp-mediated transport of PEITC cannot be excluded for low (<1 μM) incubation concentrations of PEITC and/or cells expressing higher amounts of P-gp.

Our results are consistent with previous studies in our laboratory [24] indicating that PEITC was not a P-gp substrate in MDA435/LCC6MDR1 and human intestinal Caco-2 cells. Hu and Morris [24] reported that there was no difference in intracellular concentrations of ^{14}C-PEITC in sensitive MDA435/LCC6 or P-gp-overexpressing MDA435/LCC6MDR1 cells over the concentration range of 1–100 μM, and that the intracellular concentrations of PEITC were not changed in the presence of verapamil, a P-gp and MRP inhibitor. Verapamil also had no effect on the accumulation of PEITC in Caco-2 cells which express many transporters including P-gp and MRPs. Our results differ from those of Callaway et al. [19] who reported that PEITC is rapidly exported by both P-gp and MRP1 in the form of GSH-and Cys-Gly-conjugates (dithiocarbamates); in that study, following incubation with 50 μM PEITC for 1 h, there were small but significant differences in the

intracellular concentration of PEITC and its dithiocarbamates in P-gp overexpressing cells (8226/Dox40) and the control (8226/S) and the addition of 100 μM of cyclosporin A enhanced accumulation of total ITCs significantly in 8226/Dox40 cells [19]. While these findings support the hypothesis of P-gp involvement in the efflux of PEITC and/or its metabolites, cyclosporin A is an inhibitor of both P-gp and MRP [31] and Western blot analysis of 8226/Dox40 cells revealed detectable expression of MRP1 [32]. Therefore, it is possible that the effects seen in 8226/Dox40 cells may be due to the presence of MRP1 and P-gp is not an efficient transporter of PEITC and/or its metabolites. Alternatively, the differences between the findings of the current investigation and that of Callaway et al. [29] may reflect other differences between the breast cancer cell lines (MCF7 and MDA435) and the multiple myeloma 8226/Dox40 cells used in these investigations, such as differences in metabolism. Furthermore, 8226/Dox cells may express higher levels of P-gp than the cells used in this study. The mechanism of PEITC as an inhibitor of P-gp, therefore, remains unknown. Some ITCs have been demonstrated to bind to the ATP-binding site of Na^+, K^+-ATPase [33,34]. We also found that several ITCs could inhibit ATPase of BCRP, another ABC transporter [35]. Therefore, PEITC may interact with P-gp ATPase. In addition, due to the high reactivity of ITCs with the thiol groups of proteins, PEITC may also bind to the substrate or allosteric binding sites, altering the binding of P-gp substrates, but itself is not transported by P-gp.

We and other investigators have reported that PEITC and/or its cellular metabolites represent inhibitors and substrates of MRP1 [23,24,18]. MRP1 is present in almost all the tissues in the body whereas MRP2 is mainly distributed in liver, intestine and kidney, the primary organs for drug absorption and elimination. Studies have demonstrated that MRP2 is present in substantial amounts in human heptatocytes, whereas MRP1 was only barely detectable [31]. MRP2 is localized on the apical side of epithelial membrane, in contrast to the basolateral localization of MRP1, and may play a more important role in drug absorption, distribution and elimination. Therefore, in order to understand the potential interactions between ITCs and xenobiotic compounds, we were interested in examining the interaction between PEITC and MRP2. Our results from both intracellular accumulation as well as bi-directional transport studies demonstrated that PEITC is transported by MRP2 and intracellular GSH plays an important role in the transport. Decreased intracellular GSH concentration resulted in an increased intracellular accumulation of ^{14}C-PEITC in a concentration-dependent manner. Due to the predominance of GSH conjugates of PEITC intracellularly and the fact that MRP2 transports GSH conjugates, it is highly possible that the GSH conjugate of PEITC, as well as other conjugates such as Cys-Gly-conjugates of PEITC, are actually transported by MRP2. However, at this time, we cannot exclude the possibility that PEITC itself may be transported by MRP2 in association with GSH. It is known that MRP2 can confer resistance to neutral organic drugs that are not known to be conjugated with acidic ligands such as GSH, glucuronate or sulfate, by co-transporting these drugs with free GSH [6]. Studies also have demonstrated that GSH is transported by MRP2 in an ATP-dependent manner [36]. Additionally, GSH can bind to MRP1 directly and stimulate the formation of the transition state of MRP ATPase [37]. To clarify whether PEITC itself represents a MRP2 substrate, further experiments, such as using membrane vesicles, need to be conducted.

In conclusion, we have demonstrated that PEITC (and/or its cellular metabolites) represents a substrate for MRP2, but not P-gp. The MRP2-mediated transport of PEITC is dependent on intracellular GSH levels, suggesting that GSH plays an important role in the transport of PEITC and/or its cellular metabolites. Due to the high expression of MRP2 in human intestine, liver and kidneys, PEITC may affect the bioavailability and clearance of MRP2 substrates.

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