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Membrane Transport of Dietary Phenethyl Isothiocyanate by ABCG2 (Breast Cancer Resistance Protein)

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Abstract: Isothiocyanates (ITCs) are non-nutrient constituents abundant in cruciferous vegetables and are effective in blocking carcinogenesis in a variety of tissues. ITCs permeate into cells rapidly and accumulate in cells primarily as glutathione (GSH) conjugates. We have demonstrated recently that certain ITCs are inhibitors of ABCG2 (breast cancer resistance protein, BCRP), an ATP-binding cassette transporter that plays an important role in drug absorption and disposition as well as in the development of multidrug resistance in cancer cells. The purpose of this study was to investigate the mechanisms of interactions between ITCs and BCRP and elucidate the transport of phenethyl isothiocyanate (PEITC) by BCRP. Inside-out membrane vesicles were prepared from human breast cancer BCRP-overexpressing MCF-7/MX100 and the parental MCF-7/sensitive cells. The ATPase study using 100 μM ITCs showed that ITCs are potential inhibitors of BCRP ATPase activity. The transport of ^{14}C-PEITC into BCRP-overexpressing MCF-7/MX100 cell vesicles was ATP-dependent and inhibited by fumitremorgin C (FTC), a specific inhibitor of BCRP, indicating that PEITC is a substrate for BCRP. In the control MCF-7/sensitive cell vesicles, no ATP-dependent and FTC-inhibited transport of ^{14}C-PEITC was observed. Taken together, the results of this investigation provided evidence that ITCs are potential inhibitors of BCRP ATPase and PEITC, in its unchanged form, is transported by BCRP. These data may be important in elucidating the interaction of ITCs and cellular transporters and in understanding the potential food−drug interaction.

Keywords: ABCG2; breast cancer resistance protein; isothiocyanates; phenethyl isothiocyanate; membrane transport

Introduction

The ATP-binding cassette (ABC) superfamily of membrane transporters is one of the largest protein families that include P-glycoprotein (P-gp) and the multidrug resistance associated protein (MRP) family. Present in all tissues and cell types in different amounts, ABC transporters export a large variety of structure-unrelated compounds, including anticancer drugs, xenobiotics, and endogenous compounds, in an energy-dependent manner. Therefore, they significantly contribute to the defense mechanisms used by both normal cells and malignant cells against cytotoxic compounds.[1] ABC transporters are often overexpressed in tumor tissues and thereby confer multidrug resistance to chemotherapeutic drugs, resulting in failure of cancer therapy.

ABCG2, also known as breast cancer resistance protein (BCRP), MXR, and ABCP, is an ABC transporter that contains only one ATP-binding domain and one membrane-

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spanning domain and is therefore regarded as a "half-transporter".[2] It transports many chemotherapeutic drugs and the metabolites, such as mitoxantrone, topotecan, SN-38 (the active metabolite of irinotecan), and methotrexate and its polyglutamylated forms.[3−7] Although the significance of BCRP in clinical multidrug resistance has not been completely established, an association between BCRP expression and poor response to chemotherapy has been reported.[8,9] BCRP also transports endogenous compounds such as sterols, steroids, and estrogen sulfate conjugates.[10,11] It has a wide distribution in the body, being present in liver, intestine, brain, placenta, and kidney,[12,13] and has been shown to have a transcript level higher than that of P-gp or MRP1 in human jejunum.[14] Accordingly, BCRP plays a protective role for the body against toxins. There is clear in vivo evidence that BCRP is important in the pharmacokinetics of its substrate compounds by affecting their oral absorption, elimination, and tissue distribution.[15,16] Moreover, a clinical study demonstrated that inhibiting BCRP significantly increased the oral bioavailability of its substrate drug, topotecan.[17]

Phenethyl isothiocyanate (PEITC) is abundant in many plants, especially cruciferous vegetables (e.g., broccoli, watercress, cauliflower, Brussels sprouts). Many isothiocyanates (ITCs) have been demonstrated in animal studies to be effective in blocking the initiation as well as progression of carcinogenesis.[18,19] Epidemiological studies also indicated that intake of dietary ITCs is inversely associated with the risk of lung and colon cancer development.[20−22]

Although ITCs enter cells by passive diffusion, active transport of ITCs mediated by transporters cannot be

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excluded. PEITC, benzyl ITC (BITC), and 1-naphthyl ITC (NITC) have been found to inhibit P-gp-mediated efflux of daunomycin in human breast cancer MCF-7/ADR cells and MRP1-mediated efflux of daunomycin and vinblastine in human pancreas Panc-1 cells.[23] Studies using intact cells have suggested that ITCs accumulate in cells rapidly as glutathione (GSH) conjugates and are exported by P-gp and MRP1 mainly as GSH conjugates and cysteinylglycine conjugates.[24,25] However, no direct evidence exists regarding which chemical form (parent or metabolite) is actually transported. Recently, we demonstrated that BITC, PEITC, NITC, and hexyl (HITC), phenylpropyl (PPITC), phenylbutyl (PBITC), and phenylhexyl ITC (PHITC) (Figure 1) inhibited BCRP-mediated transport of mitoxantrone in BCRP-overexpressing human breast cancer MCF-7/MX100 and human large cell lung carcinoma NCI-H460/MX20 cells; moreover, drug accumulation studies in NCI-H460/MX20 cells demonstrated that PEITC and/or cellular substrates represent substrates of BCRP.[26] In the present study, we further investigated the mechanisms of the interaction between ITCs and BCRP by examining the effect of ITCs on BCRP ATPase and clarifying whether PEITC or its cellular metabolites are transported by BCRP using cell membrane vesicles.

Experimental Section

Materials. BITC, NITC, PEITC, mitoxantrone, creatine kinase, phosphocreatine, ATP disodium, and AMP sodium were purchased from Sigma-Aldrich (St. Louis, MO). PPITC and PBITC were purchased from LKT Laboratories (St. Paul, MN). PHITC was a kind gift from National Cancer Institute—Chemopreventive Division (Bethesda, MD). RPMI 1640 medium, fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin-streptomycin, and trypsin were supplied by Invitrogen (Buffalo, NY). ^{14}C-PEITC was synthesized in our laboratory with a specific activity of 0.04994 mCi/mg and a concentration of 1 mCi/mL in ethanol. The mitoxantrone-selected BCRP-overexpressing human breast cancer MCF-7/MX100 cells and the parental MCF-7/sensitive cells, the mitoxantrone-selected BCRP-overexpressing human large cell lung carcinoma NCI-H460/MX20 cells, and fumitremorgin C (FTC) were generous gifts from Dr. Susan E. Bates (National Cancer Institute, Bethesda, MD).

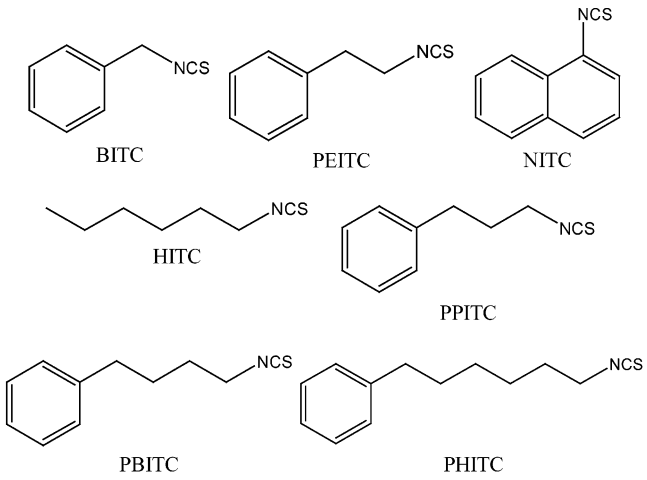

Figure 1. Chemical structures of ITCs.

Cell Culture. MCF-7/sensitive, MCF-7/MX100, and NCI-H460/MX20 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 50 IU/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2/95% air. Mitoxantrone of 100 and 20 nM was supplemented in the medium for the culture of MCF-7/MX100 and NCI-H460/MX20 cells, respectively. Cells were maintained in 75 cm^2 plastic culture flasks with culture medium replaced every 2 days, and 0.25% trypsin-EDTA was used to detach cells for subculture when 90% confluence was reached.

Preparation of Membrane Vesicles. BCRP-overexpressing MCF-7/MX100 and parental MCF-7/sensitive cells were seeded in 150 cm^2 plastic culture flasks. Upon 90% confluence, 20 flasks of cells were washed twice with PBS, scraped to detach, and combined for the vesicle preparation. BCRP-containing and BCRP-negative inside-out membrane vesicles were isolated from MCF-7/MX100 and MCF-7/sensitive cells, respectively, by a nitrogen cavitation method as described by Volk and Schneider.[7] Vesicle preparations were analyzed for protein concentration by a BCA assay[27] using an assay kit (Pierce Biotechnology, Inc., Rockford, IL) and characterized for BCRP expression by Western blot analysis as described previously.[28] The prepared membrane vesicles were stored as 50 μL aliquots at −80 °C.

ATPase Activity Assay. BCRP ATPase activity was measured on the basis of a colorimetric reaction between

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ammonium molybdate and liberated inorganic phosphate from ATP in the presence of ascorbic acid as previously described.[29] Ten microgram membrane vesicles were incubated with 100 μM ITCs, solvent control (0.2% DMSO), or 10 μM mitoxantrone (positive control). ATPase activity was determined by the difference of the phosphate level between a 0 min and a 20 min incubation period and expressed as the rate of phosphate release per milligram of membrane protein. BCRP ATPase activity was estimated as orthovanadate-sensitive ATPase activity by subtracting the sample ATPase activity in the presence of 1 mM orthovanadate (Sigma-Aldrich, St. Louis, MO) from that in the absence of orthovanadate. Control experiments using phosphate standards found that 100 μM ITCs or 10 μM mitoxantrone did not interfere with the colorimetric assay.

Drug Accumulation Studies in Vesicles. Vesicle drug accumulation studies were performed according to Volk and Schneider with modifications.[7] Briefly, reaction mixtures of 100 μL containing 0.1 mg/mL creatine kinase, 10 mM phosphocreatine, 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 250 mM sucrose, 4 mM ATP or AMP, and 25 μM ^{14}C-PEITC were preincubated at 37 °C in glass test tubes. The transport was initiated by the addition of 5 μg of membrane vesicles and stopped by the addition of 1 mL of ice-cold buffer containing 10 mM Tris-HCl (pH 7.5) and 250 mM sucrose (TS buffer) after a specified incubation period at 37 °C. One milliliter of reaction mixture was immediately transferred onto a 0.45 μm nitrocellulose filter membrane (Millipore) to separate the vesicles and free drugs. The membranes used had been presoaked in 250 μM PEITC in TS buffer overnight to minimize nonspecific binding of PEITC to the membrane. The membrane was immediately rinsed twice with 5 mL of ice-cold TS buffer, dissolved in 1 mL of 2-methoxyethanol, and vortexed for 45 s. Radioactivity present on the membrane represents the drug amount remaining in the vesicles. For the inhibition study, ^{14}C-PEITC accumulation after a 15 min incubation was determined in the presence or absence of 10 μM FTC.

Statistical Analysis. Statistical evaluation was conducted using a one-way ANOVA followed by Dunnett's or Tukey's post hoc test, and $p < 0.05$ was considered significant.

Results

Expression of BCRP in Membrane Vesicles. To use prepared membrane vesicles to explore the interaction between ITCs and BCRP, we first characterized the expression of BCRP in the vesicles by Western blot analysis. The protein loading amounts of cell lysates and vesicles were 50 and 10 μg, respectively. Both mitoxantrone-selected MCF-7/MX100 and NCI-H460/MX20 cells overexpress wild-type BCRP[30] with no detectable expression of P-gp or MRP1 by Western blot analysis, and there is no detectable expression of BCRP, P-gp, or MRP1 in the parental MCF-7/sensitive cells by Western blot analysis.[28] In addition, no MRP2 was detected by Western blot analysis in either MCF-7/MX100 or MCF-7/sensitive cells.[31] As expected, the membrane vesicles prepared from BCRP-overexpressing MCF-7/MX100 cells have pronounced expression of BCRP, whereas negligible BCRP was detected in the vesicles obtained from the parental cell line MCF-7/sensitive (Figure 2).

Figure 2. Western blot analysis of BCRP expression. Cell lysates (50 μg) of MCF-7/sensitive (lane 1), MCF-7/MX100 cells (lane 2), and NCI-H460/MX20 (lane 3) and membrane vesicles (10 μg) prepared from MCF-7/sensitive (lane 4) and MCF-7/MX100 (lane 5) cells were separated and analyzed for BCRP expression by Western blot analysis.

Table 1. Effects of ITCs on BCRP ATPase Activity [nmol of P_i (mg of protein)$^{-1}$ min^{-1}] in MCF-7/Sensitive and MCF-7/MX100 Cell Membranes

	MCF7/sensitve	MCF7/MX100
control	6.33 ± 4.91	10.25 ± 9.29
mitoxantrone (10 μM)	10.61 ± 18.48	57.29 ± 24.38[a]
BITC (100 μM)	10.14 ± 9.22	−17.89 ± 13.26
PEITC (100 μM)	17.17 ± 9.23	−13.37 ± 11.65
NITC (100 μM)	14.31 ± 10.26	−15.57 ± 11.79
PBITC (100 μM)	11.02 ± 7.90	−15.26 ± 12.72
PPITC (100 μM)	21.46 ± 6.87	−7.92 ± 12.06
PHITC (100 μM)	22.31 ± 19.44	6.72 ± 7.78

[a] $p < 0.01$ compared with the control by one-way ANOVA followed by Dunnett's post hoc test; data are expressed as mean ± SD from one typical experiment with triplicate measurements.

Effects of ITCs on BCRP ATPase Activity. To understand the potential mechanisms of ITCs as BCRP inhibitors, we evaluated the effects of ITCs on BCRP ATPase activity using BCRP-overexpressing MCF-7/MX100 and the control MCF-7/sensitive membrane vesicles. The ITCs investigated are BITC, PEITC, NITC, PBITC, and PHITC, which are able to inhibit BCRP-mediated transport of mitoxantrone.[26] As shown in Table 1, the BCRP ATPase basal levels in vesicles prepared from MCF-7/MX100 and MCF-7/sensitive cells were 10.25 ± 9.29 and 6.33 ± 4.91 nmol of P_i (mg of protein)$^{-1}$ min^{-1}, respectively. In MCF-7/MX100 vesicles, 10 μM mitoxantrone, a known BCRP substrate able to stimulate ATPase activity,[32] increased ATPase activity 5.6-fold (57.29 ± 24.38 nmol of P_i (mg of protein)$^{-1}$ min^{-1}, $p < 0.01$). However, the MCF-7/MX100 cell vesicles treated by ITCs have little or no orthovanadate-sensitive ATPase activity. Although not statistically significant, ITC treatment reduced ATPase activity in BCRP-expressing MCF-7/MX100 membrane vesicles compared to the control (treated with vehicle only), suggesting that ITCs may inhibit BCRP ATPase activity. In the MCF-7/sensitive cell vesicles that have no BCRP expression, none of the tested ITCs affected the orthovanadate-sensitive ATPase activity significantly.

^{14}C-PEITC Accumulation into Membrane Vesicles. To elucidate whether the parent form of PEITC is transported by BCRP in an ATP-dependent manner, the accumulation of ^{14}C-PEITC into inside-out membrane vesicles derived

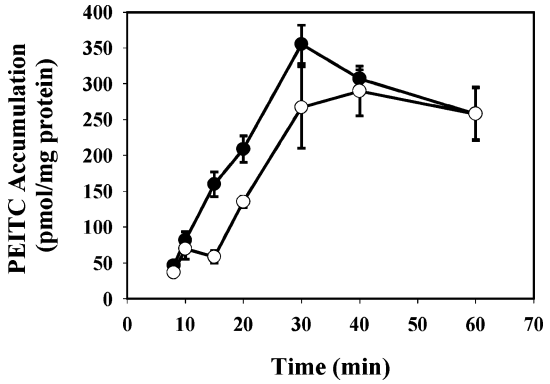

Figure 3. PEITC accumulation in BCRP-expressing MCF-7/MX100 cell membrane vesicles. ^{14}C-PEITC accumulation into membrane vesicles prepared from MCF-7/MX100 cells in the presence of ATP (●) or AMP (○) was measured over time. The data are expressed as mean ± SD, $n = 3$.

from BCRP-overexpressing MCF-7/MX100 and parental MCF-7/sensitive cells was examined in the presence of ATP or AMP. Unlike intact cells, cell membrane vesicles do not contain metabolic enzymes or GSH; therefore, formation of cellular metabolites of PEITC is expected to be minimal. We first characterized the kinetic profile of ^{14}C-PEITC accumulation into BCRP-expressing inside-out membrane vesicles generated from MCF-7/MX100 cells. PEITC accumulation into MCF-7/MX100 cell vesicles increased over time and reached a plateau at 30 min (Figure 3). The accumulated amounts of PEITC were consistently higher in the presence of ATP than those in the presence of AMP over time, except at 60 min when ATP may be depleted. This ATP-dependent PEITC accumulation suggests that PEITC itself is actively transported. To further confirm that the transport is mediated by BCRP, we measured the accumulation of PEITC into vesicles in the presence and absence of FTC, a potent and specific BCRP inhibitor.[33,34] We chose a 15 min incubation for the study because the ATP-dependent transport of PEITC was linear at this time point (Figure 3). In BCRP-overexpressing MCF-7/MX100 cell vesicles and using the amount of accumulated PEITC in the presence of AMP as the basal value (100 ± 9.4%), the accumulated PEITC in the presence of ATP was significantly higher (136.0 ± 18.5%, $p < 0.05$), indicating again that transport of PEITC is ATP-dependent (Figure 4A). Furthermore, compared to the absence of FTC, the presence of 10 μM FTC significantly decreased ATP-dependent PEITC accumulation 1.7-fold (from 136.0 ± 18.5% to 81.3 ± 24.1%, $p < 0.01$), indicating that PEITC transport was inhibitable by a BCRP inhibitor. On the other hand, when AMP was present, FTC had no effect on PEITC accumulation into MCF-7/MX100 cell vesicles (Figure 4A), suggesting that only energy-dependent transport of PEITC was inhibited by a BCRP inhibitor. In the negative control MCF-7/sensitive cell vesicles, there was no difference for PEITC accumulation among all the tested groups (in the presence of ATP or AMP with the presence or absence of 10 μM FTC), indicating that the transport of PEITC is BCRP specific (Figure 4B). Taken

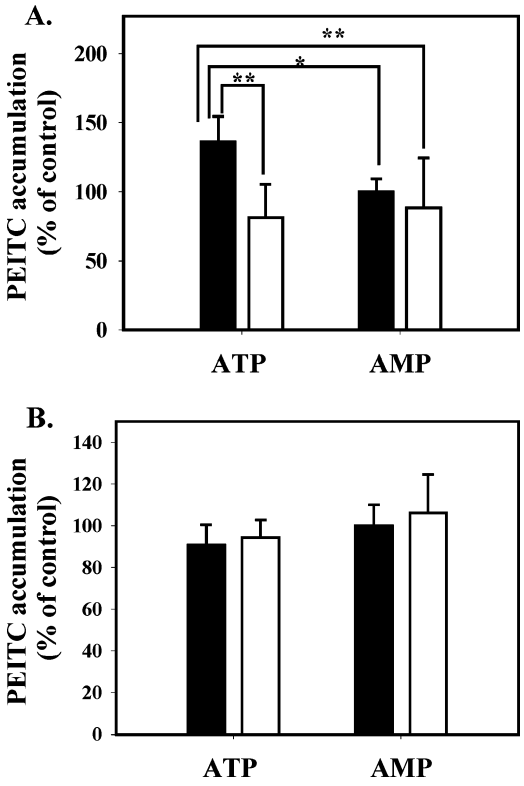

Figure 4. PEITC accumulation in MCF-7/MX100 and MCF-7/sensitive cell membrane vesicles with the presence of FTC. ^{14}C-PEITC accumulation in membrane vesicles prepared from BCRP-overexpressing MCF-7/MX100 (A) and MCF-7/sensitive (B) cells was measured in the presence of ATP or AMP as well as in the absence (solid bar) or presence (empty bar) of 10 μM FTC, a specific and potent BCRP inhibitor. The data are presented as percent of control, where control is the accumulation amount of ^{14}C-PEITC in the cell membrane vesicles treated with AMP in the absence of FTC. Statistical analysis was conducted by one-way ANOVA followed by Tukey's post hoc test; $^{**}p < 0.01$, $^{*}p < 0.05$, $n = 6$ or 7 for MCF-7/MX100 vesicles and $n = 3$ for MCF-7/sensitive vesicles.

together, PEITC itself is transported in an ATP-dependent manner by BCRP.

Discussion

BCRP is an ATPase and capable of vanadate-dependent adenine nucleotide trapping.[32] It has high basal ATPase activity compared with that of P-gp. BCRP substrates, mitoxantrone, prazosin, and flavopiridol, are good stimulators, and its inhibitor FTC is a strong inhibitor of BCRP ATPase activity.[33,34] Due to binding to thiol groups on proteins, ITCs have been successfully applied in studying the active site structure of Na^+,K^+-ATPase. Fluorescein 5′-ITC inactivates Na^+,K^+-ATPase through reacting at the ATP hydrolysis site,[35] and 1-pyreneisothiocyanate binds to Na^+,K^+-ATPase and thereby makes the ATP-binding site of Na^+/K^+-ATPase more rigid and rotationally immobilized.[36] Therefore, affecting the ATPase activity of BCRP may be one potential mechanism for ITCs to inhibit BCRP-

mediated transport. We measured the orthovanadate-sensitive ATPase activity in membrane vesicles derived from MCF-7/MX100 and MCF-7/sensitive cells and found that 100 μM ITCs potentially inhibited BCRP ATPase activity, although the effects were not statistically significant. The failure to observe statistical significance may be a consequence of the limit of assay sensitivity, as the basal BCRP ATPase activity in the prepared vesicles was low and further inhibition may be difficult to detect due to experimental variability.

PEITC permeates into cells freely by passive diffusion and rapidly reacts with thiol groups by nonenzymatic reaction with cysteine residues and enzymatic conjugation with GSH catalyzed by GSH S-transferases.[37] The intracellular accumulation of the GSH conjugate of PEITC is maximal after about 30 min in cultured cells; on the other hand, the conjugate is labile and fragments spontaneously to PEITC and GSH under physiological conditions with an estimated half-life of 44 min.[37] Although the majority of intracellular ITCs are present in the GSH conjugate forms, transport studies using intact cells that measure the change of extracellular and intracellular concentrations of different PEITC conjugates cannot provide direct evidence of transport of these conjugates. First, conjugation of PEITC and GSH is highly reversible and free PEITC can be formed inside cells. Thus, one cannot preclude the contribution of free PEITC to active transport. Second, PEITC has high protein binding and is expected to bind to cellular proteins extensively, resulting in high intracellular concentration. Therefore, the change in total (both free and bound) concentration of one chemical entity, that is usually measured, does not represent the real transport behavior of that entity since only free drug is transported. Third, after being exported, the conjugates can hydrolyze back to the free ITC to re-enter cells, making the transport situation more complicated. Membrane vesicles represent a simplified system with minimal GSH and metabolic enzymes and were used in this study to clarify the actual chemical entity of PEITC/metabolites that is transported.

The kinetic study of transport of ^{14}C-PEITC in membrane vesicles demonstrated that PEITC accumulated in BCRP-expressing inside-out membrane vesicles in a time-dependent and ATP-dependent manner, indicating that PEITC is actively transported. However, the compound also has high accumulation in the presence of AMP. This may be due to high permeability of the compound into cell vesicles. High nonspecific binding of PEITC to the nitrocellulose filter membrane could be another reason. Although the filter membranes have been presoaked with PEITC, the soaking solution cannot be higher than 250 μM due to the limited solubility of PEITC. Nevertheless, in the presence of ATP, PEITC exhibited a saturable transport into BCRP-expressing vesicles over time. Moreover, the inhibition studies showed that the active transport of PEITC can be inhibited by a BCRP inhibitor FTC, further demonstrating that PEITC is transported by BCRP. Previously, we observed that total intracellular radioactivity, including both PEITC and its GSH conjugate, in BCRP-overexpressing NCI-H460/MX20 cells was significantly lower than that in the parental NCI-H460 cells; moreover, 10 μM FTC dramatically enhanced the intracellular radioactivity in NCI-H460/MX20 cells but not in NCI-H460 cells.[26] The current study indicated that the phenomenon is due, at least in part, to the transport of unchanged (nonmetabolized) PEITC by BCRP. We found that substantially reduced intracellular GSH levels due to pretreatment with buthionine sulfoximine, a GSH synthesis inhibitor, did not enhance the intracellular accumulation of radioactivity in BCRP-overexpressing MCF-7/MX100 cells incubated with ^{14}C-PEITC (data not shown), suggesting that GSH is not critical in the BCRP transport of PEITC. Therefore, the GSH conjugate of PEITC may not be transported by BCRP or may be only a poor substrate of BCRP; however, further investigation is needed to confirm the hypothesis.

In conclusion, organic ITCs are BCRP inhibitors and one potential mechanism involves inhibition of BCRP ATPase activity by ITCs. In addition, PEITC in the unchanged form is transported by BCRP; therefore, ITCs may represent substrates of BCRP. Understanding the mechanisms underlying interactions between ITCs and BCRP may be important in elucidating potential food−drug interactions.

Abbreviations Used

ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; BITC, benzyl isothiocyanate; FTC, fumitremorgin C; GSH, glutathione; HITC, hexyl isothiocyanate; ITC, isothiocyanate; MRP, multidrug resistance associated protein; NITC, 1-naphthyl isothiocyanate; PBITC, phenylbutyl isothiocyanate; PEITC, phenethyl isothiocyanate; P-gp, P-glycoprotein; PHITC, phenylhexyl isothiocyanate.

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