Effect of Organic Isothiocyanates on Breast Cancer Resistance Protein (ABCG2)-Mediated Transport

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Purpose. To investigate the effect of organic isothiocyanates (ITCs), dietary compounds with chemopreventive activity, on breast cancer resistance protein (BCRP)-mediated transport.

Methods. The effect of 12 ITCs on the cellular accumulation of mitoxantrone (MX) was measured in both BCRP-overexpressing and BCRP-negative human breast cancer (MCF-7) and large cell lung carcinoma (NCI-H460) cells by flow cytometric analysis. The ITCs showing activity in MX accumulation were examined for their effect on MX cytotoxicity, and the intracellular accumulation of radiolabeled phenethyl isothiocyanate (PEITC) was measured in both BCRP-overexpressing and BCRP-negative NCI-H460 cells.

Results. ITCs significantly increased MX accumulation and reversed its cytotoxicity in resistant cells, but had a small or no effect in sensitive cells. The effects of ITCs on MX accumulation and cytotoxicity were ITC-concentration dependent. Significant increases in MX accumulation were observed at ITC concentrations of 10 or 30 μM, and significant reversal of MX cytotoxicity was generally observed at ITC concentrations of 10 μM. Intracellular accumulation of radiolabeled PEITC in BCRP-overexpressing cells was significantly lower than that in BCRP-negative cells and was increased significantly by the BCRP inhibitor fumitremorgin C (FTC).

Conclusions. Certain ITCs are BCRP inhibitors and PEITC and/or its cellular metabolite(s) may represent BCRP substrates, suggesting the potential for diet-drug interactions.

KEY WORDS: breast cancer resistance protein; isothiocyanates; membrane transport.

INTRODUCTION

Transmembrane transporters of the ATP-binding cassette (ABC) superfamily act as efflux pumps and result in decreased intracellular concentrations of various structurally and functionally unrelated compounds. Often overexpressed in tumor cells, these transporters actively export anticancer drugs, decreasing intracellular concentrations below therapeutic thresholds and conferring multidrug resistance (MDR). ABC transporters are also present in many normal tissues such as intestine, blood-brain barrier, liver, and kidney, where they serve a protective role, through the ATP-dependent efflux of xenobiotics and toxins. As a consequence, ABC transporters play an important role in drug absorption, disposition and elimination (1).

Breast cancer resistance protein (BCRP), also known as ABCG2, mitoxantrone resistant protein (MXR) or ABC transporter in placenta (ABCP), is a half-size ABC transporter with only one N-terminal ATP binding domain and one C-terminal transmembrane region with six transmembrane segments (2). BCRP mediates resistance to chemotherapeutic agents including mitoxantrone (MX), topotecan, doxorubicin (DOX) and SN-38 (the active metabolite of CPT-11) (3–6). BCRP also transports sterols, steroids and estrogen sulfate conjugates, suggesting its physiologic role in humans (7,8). Localized in the plasma membrane, BCRP is widely present in human tumor tissues and some studies have reported an association between BCRP overexpression and poor responses in clinical chemotherapy (9,10). BCRP is also expressed in a wide variety of normal tissues including placenta, liver, intestine, colon, lung, kidney, adrenal, sweat glands, and the endothelia of veins and capillaries (2). Localized in the apical membranes of intestinal epithelium and the biliary canalicular membranes, BCRP affects the intestinal uptake of orally administered drugs and their hepatobiliary excretion (11). In fact, BCRP and multidrug resistance-associated protein 2 (MRP2) transcripts are more abundant than MDR1 (for P-glycoprotein) or MRP1 transcripts in normal human jejunum (12). Clinical studies have demonstrated that inhibition of intestinal BCRP improves the oral bioavailability of topotecan in humans (13); and mice lacking Bcrp1/Abcg2 were reported to be extremely sensitive to diet-induced phototoxicity by pheophorbide a, suggesting a greater bioavailability of pheophorbide a in bcrp1-deficient mice (14).

Organic isothiocyanates (ITCs) (R-N=C=S) occur in cruciferous vegetables as glucosinolates, which can be hydrolyzed to ITCs by myrosinase released from damaged plant cells. ITCs are of interest since numerous studies have demonstrated that more than 25 natural and synthetic ITCs block the carcinogenic effects of a variety of chemically different types of carcinogens in over 10 target sites (15). Human glucosinolate consumption has been assessed to be as high as 300 mg/d and consuming normal amounts of vegetables such as watercress or broccoli releases milligram amounts of ITCs (16). Besides the food sources, health products containing ITCs as food supplements have emerged in the market recently, as the result of increasing public attention to the health beneficial property of ITCs. Accordingly, high ITC concentrations in the intestine may be achieved, and one may anticipate food-drug interactions.

Our laboratory has demonstrated that certain ITCs are weak inhibitors of P-glycoprotein (P-gp)- and MRP1-mediated transport (17,18). Whether ITCs have effects on BCRP, which may be important in both MDR and intestinal drug absorption, is not known. Therefore, the present study was designed to examine the effect of ITCs on BCRP-mediated transport. Twelve dietary and synthetic ITCs were evaluated for their effects on the cellular accumulation and cytotoxicity of MX in both BCRP-overexpressing and BCRP-negative

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ABBREVIATIONS: ABC, ATP-binding cassette; AITC, allyl isothiocyanate; BCRP, breast cancer resistance protein; BITC, benzyl isothiocyanate; FTC, fumitremorgin C; GSH, glutathione; HITC, hexyl isothiocyanate; ITC, isothiocyanate; MDR, multidrug resistance; MRP1, multidrug resistance-associated protein 1; MRP2, multidrug resistance-associated protein 2; MX, mitoxantrone; NITC, 1-naphthyl isothiocyanate; PBITC, phenylbutyl isothiocyanate; PEITC, phenethyl isothiocyanate; P-gp, P-glycoprotein; PHITC, phenylhexyl isothiocyanate; PITC, phenyl isothiocyanate; PPITC, phenylpropyl isothiocyanate; SRB, sulforhodamine B.

cells. The compounds evaluated were allyl- (AITC), benzyl- (BITC), hexyl- (HITC), 1-naphthyl- (NITC), phenyl- (PITC), phenethyl- (PEITC), phenylhexyl- (PHITC), phenylpropyl- (PPITC), phenylbutyl-isothiocyanate (PBITC), sulforaphane, erucin and erysolin. The intracellular accumulation of radiolabeled PEITC was also measured in both BCRP-overexpressing and BCRP-negative cells, to determine if PEITC is a substrate for BCRP.

MATERIALS AND METHODS

Materials

AITC, BITC, HITC, NITC, PITC, PEITC, erysolin, MX and sulforhodamine B (SRB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sulforaphane, PBITC, and PPITC were purchased from LKT Laboratories (St. Paul, MN, USA). Erucin was purchased from ICN Biomedicals, Inc. (Irvine, CA, USA). PHITC was a gift from National Cancer Institute-Chemopreventive Division (Bethesda, MD, USA). GF120918 was obtained from GlaxoSmithKline Inc. (Research Triangle Park, NC, USA). Radiolabeled ^{14}C-PEITC (specific activity 8.1 mCi/mmol, chemical purity >97% by HPLC) was synthesized and characterized by Qi Wang in our laboratory. RPMI 1640, fetal bovine serum (FBS), and phosphate buffered saline (PBS) were supplied by Gibco BRL (Buffalo, NY, USA). MCF-7/sensitive and the resistant MCF-7/MX100 cells, NCI-H460 and the resistant NCI-H460/MX20 cells, as well as fumitremorgin C (FTC) were kindly provided by Dr. Susan E. Bates (National Cancer Institute, Bethesda, MD, USA). Biodegradable liquid scintillation cocktail was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Cell Culture

MCF-7/sensitive, MCF-7/MX100, NCI-H460, and NCI-H460/MX20 cells were grown in RPMI 1640 supplemented with 10% FBS, 50 IU/ml penicillin and 50 μg/ml streptomycin. MX of 100 and 20 nM were supplemented for MCF-7/MX100 and NCI-H460/MX20 cells, respectively. Expression of BCRP was confirmed by Western blot analysis as described previously (19). Cells were incubated in 75 mm^2 plastic culture flasks at 37°C supplemented with 5% CO_2/95% air.

MX Accumulation Assay

The MX accumulation assay was performed as described previously (19). Briefly, after being trypsinized and washed with FBS-free RPMI 1640, 1 ml of cells (~10^6 cells) were exposed with various concentrations of ITCs, the vehicle (0.1% DMSO), or 10 μM FTC (as a positive control) at 37°C for 15 min, followed by the addition of 3 μM of MX for 30 min. The accumulation was stopped by adding 3 ml of ice-cold PBS, followed by centrifugation. After cells were washed with ice-cold PBS two more times, the intracellular level of MX was determined by measuring MX fluorescence using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with a standard argon laser for 488-nm excitation and a 670 nm bandpass filter. The accumulation of MX was expressed as percent of the control, where control represented cells treated with the vehicle.

MX Cytotoxicity Assay

The cytotoxicity assay was performed as described previously with minor modifications (19). Briefly, cells were trypsinized and plated in 96-well plates at a density of 5,000 (for NCI-H460) or 10,000 (for NCI-H460/MX20) cells per well. After 48-h attachment, fresh medium containing a serial dilution of MX as well as the specified concentrations of ITCs, the vehicle (0.1% DMSO), or 10 μM FTC (as a positive control), was added to the plates. The MX concentration range used for NCI-H460 and NCI-H460/MX20 cells were 0–300 and 0–1000 μM, respectively. Cells were incubated for another 24 h and cell growth in each well was determined by a SRB assay (20). The absorbance values at 570 nm (OD_{570}) from the SRB assay represent the cell number in each well of the plates. The IC_{50} value, the concentration required to inhibit 50% of cell growth, was determined by fitting cell growth curves using WinNonlin Professional 2.1 (Pharsight, Mountain View, CA). The fitting equation was

$$F = 100 \times \left(1 - \frac{I_{max} \cdot C^{\gamma}}{IC_{50}^{\gamma} + C^{\gamma}}\right),$$

where C is the concentration of MX and F is cell survival fraction. F was calculated as 100 times the ratio of the cell growth [OD_{570} – OD_{570} (maximal concentration)] to the maximum cell growth [$OD_{570}(0)$ – OD_{570} (maximal concentration)], where $OD_{570}(0)$ and OD_{570} (maximal concentration) are the absorbance values from cells treated without and with the maximal concentration of MX, respectively. Quadruplicate measurements were performed in each experiment.

^{14}C-PEITC Accumulation Assay

The accumulation of ^{14}C-PEITC was evaluated as previously described with minor modifications (17). Cells were trypsinized and seeded on 35 mm^2 dishes at a density of 5 × 10^5 cells per dish and used two days later. Growth medium was removed from monolayer cells and cells were washed twice with sodium buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, pH 7.4). One ml of sodium buffer containing specified concentrations of ^{14}C-PEITC (supplemented with cold PEITC) with or without other modulators was added to the dish and incubated for 1 h. FTC and GF120918, inhibitors of BCRP, were used as positive controls. The accumulation was stopped by aspirating the incubation buffer and washing the cells three times with ice-cold stop solution (137 mM NaCl, 14 mM Tris-base, pH 7.4). One ml 0.3 N NaOH-1% SDS was added to each dish, and cell lysates were collected after an hour. The radioactivity was measured by a liquid scintillation counter (1900 CA, Tri-Carb liquid scintillation analyzer, Packard Instruments Co., Meridan, CT) and the protein concentration was determined by a BCA assay using a commercially available assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) (21).

Statistical Analysis

Statistical evaluation was performed using a one-way ANOVA followed by Dunnett's *post hoc* test or using a student's *t* test. Significant differences were considered when $p < 0.05$.

RESULTS

Effect of ITCs on the Intracellular Accumulation of MX

MX is a specific substrate for BCRP with high binding affinity. To examine whether organic ITCs modulate BCRP,

a MX accumulation study was performed in BCRP-overexpressing cells (MCF-7/MX100 and NCI-H460/MX20 cells) as well as the corresponding BCRP-negative cells (MCF-7/sensitive and NCI-H460 cells). The four cell lines have been characterized in our laboratory previously (19) and both MCF-7/MX100 and NCI-H460/MX20 cells express wild-type BCRP (482R) (22). FTC, a specific BCRP inhibitor, was used as a positive control in the study. The fluorescence values of all the tested ITCs at the settings used in the study are negligible (data not shown). After cells were exposed to 50 μM ITCs and 3 μM MX, intracellular accumulation of MX in MCF-7/MX100 cells was significantly increased by BITC, PEITC, NITC, PBITC, PPITC, HITC and PHITC by 3.5- to 5.8-fold ($p < 0.001$), suggesting their inhibition of MX efflux in the MX-selected resistant cells (Fig. 1A). MX accumulation was increased 5-fold by FTC (10 μM) ($p < 0.001$) in MCF-7/MX100 cells, but was not altered in MCF-7/sensitive cells, which lack BCRP, based on Western blot analysis (19) (Fig. 1A). BITC, PEITC, PBITC, PPITC and PHITC produced the greatest effects and were able to increase intracellular MX accumulation by more than 5-fold, comparable to that caused by FTC. The seven ITCs showing effects in MCF-7/MX100 cells also increased MX accumulation in MCF-7/sensitive cells ($p < 0.05$ for BITC and $p < 0.001$ for the other ITCs), but to a much smaller extent (Fig. 1A). AITC, sulforaphane, erucin, and erysolin did not alter MX accumulation in MCF-7/MX100 and MCF-7/sensitive cells; PITC did not alter MX accumulation in MCF7/MX100 cells but had a small effect in the sensitive cells ($p < 0.001$) (Fig. 1A). The ITCs active in MCF-7/MX100 cells also significantly increased MX accumulation in the MX-selected NCI-H460/MX20 cells, although to a smaller extent (2.4- to 3.1-fold; $p < 0.001$) (Fig. 1B). In NCI-H460/MX20 cells, FTC (10 μM) increased the intracellular MX level by 2.6-fold ($p < 0.001$); in NCI-H460 cells, the BCRP-negative cells, accumulation of MX in the presence or absence of FTC was unchanged (Fig. 1B). As in MCF-7/MX100 cells, the highest intracellular MX levels in NCI-H460/MX20 cells were observed for those treated by PHITC, PEITC and PBITC, and were 3-fold greater than MX accumulation in cells not treated with ITCs. BITC ($p < 0.001$), PEITC ($p < 0.05$) and PPITC ($p < 0.001$) increased MX accumulation significantly, but slightly, in the NCI-H460 cells. AITC, PITC, sulforaphane, erucin and erysolin had no effect in both NCI-H460/MX20 and NCI-H460 cells (Fig. 1B). A significant correlation was seen for MX accumulation in MCF-7/MX100 cells and that in NCI-H460/MX20 cells ($r^2 = 0.86$; $p < 0.01$; Fig. 1C).

Concentration-Dependent Effect of ITCs on the Intracellular Accumulation of MX

To determine whether the effects of ITCs on MX accumulation were concentration dependent, accumulation studies using a series of ITC concentrations (1, 5, 10, 30, and 50 μM) were performed. BITC, PEITC, NITC, HITC, PBITC, PPITC and PHITC, the ITCs that significantly increased MX accumulation at a concentration of 50 μM, were tested in both MCF-7/MX100 and NCI-H460/MX20 cells. With an increase in ITC concentration, the intracellular accumulation of MX generally increased correspondingly, demonstrating dose dependence in both cell lines (Fig. 2). In MCF-7/MX100 cells, all the tested ITCs had significant effects on MX accumulation at 30 μM ($p < 0.001$). In addition, 10 μM of NITC ($p < 0.001$) or

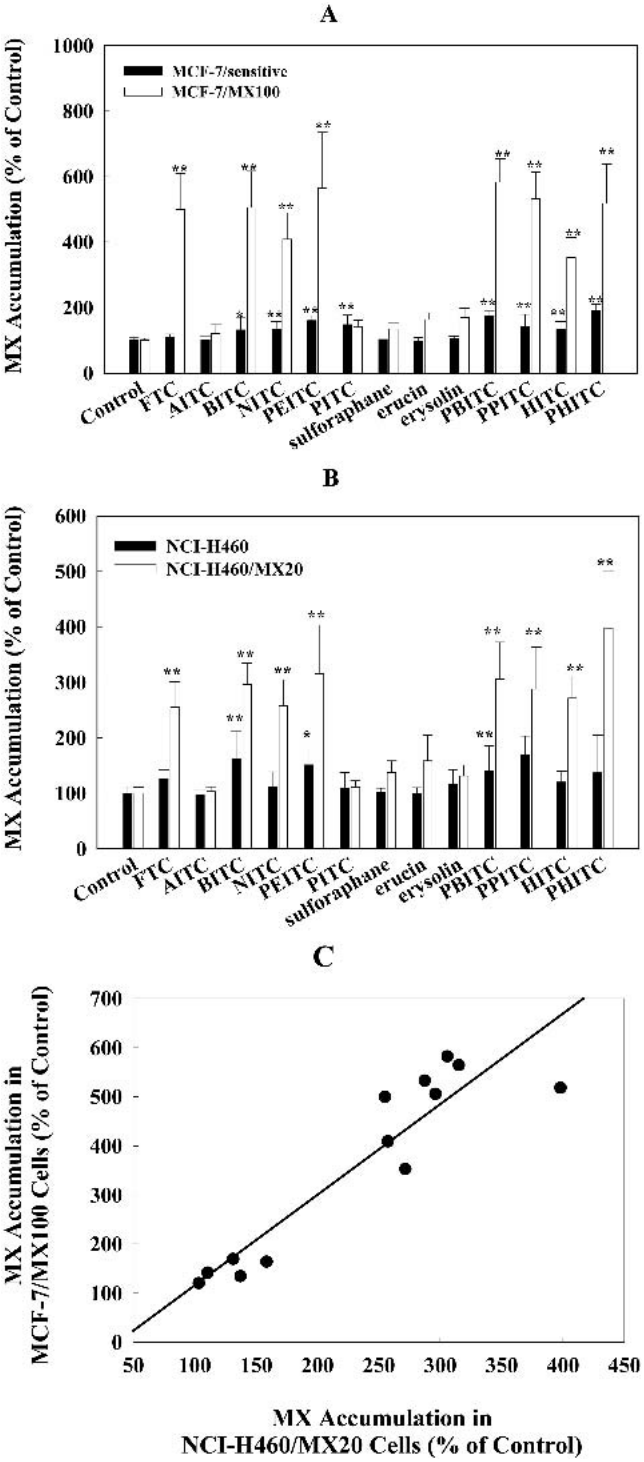

Fig. 1. Effect of ITCs on the intracellular accumulation of MX in the parental and MX-selected MCF-7 and NCI-H460 cells. The 30-min accumulation of 3 μM MX was measured in the absence (0.1% DMSO) or presence of various ITCs (50 μM) in MCF-7/sensitive and MCF-7/MX100 cells (A) as well as in NCI-H460 and NCI-H460/MX20 cells (B). The intracellular accumulation of MX is expressed as percent of control, where control represents the MX accumulationin the absence of ITCs. Data are expressed as mean ± SD, n = 9. Statistical analysis was conducted by a one-way ANOVA followed by Dunnett's *post hoc* test; *$p < 0.05$; **$p < 0.001$. (C) Relationship between MX accumulation in MCF-7/MX100 and in NCI-H460/MX20 cells. Each point represents the mean value of the percent of control (n = 9) and the line was generated from linear regression with an r^2 of 0.86 ($p < 0.01$).

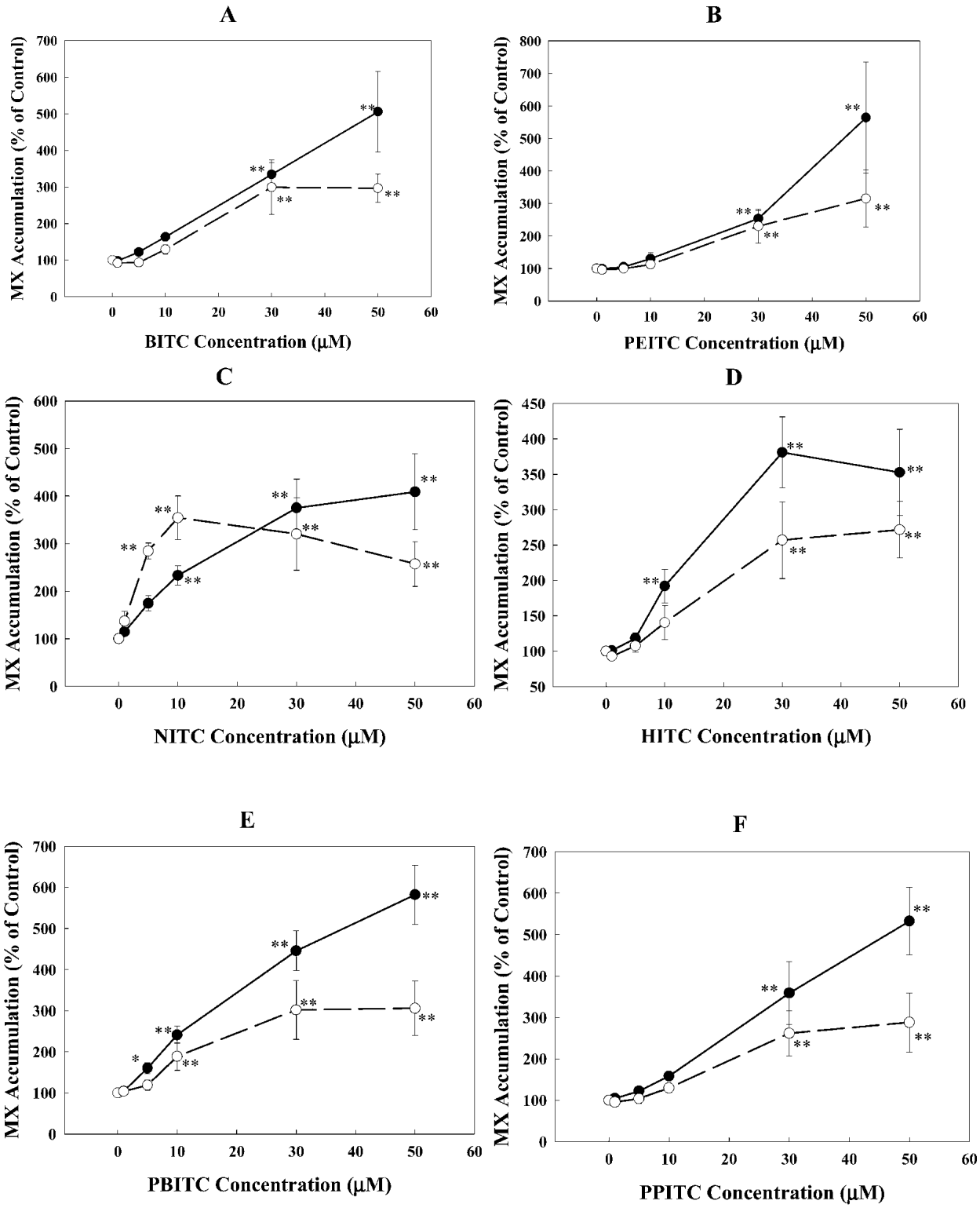

Fig. 2. Concentration-dependent effect of ITCs on MX accumulation in MCF-7/MX100 and NCI-H460/MX20 cells. Cells were incubated with 1, 5, 10, 30, and 50 μM of BITC (A), PEITC (B), NITC (C), HITC (D), PBITC (E), PPITC (F), or PHITC (G). The intracellular accumulation of MX was determined by flow cytometric analysis and expressed as percent of control (in the absence of ITCs) in MCF-7/MX100 cells (●, solid line) or NCI-H460/MX20 cells (○, dashed line). The data are expressed as mean ± SD, n = 9. Statistical analysis was conducted by a one-way ANOVA followed by Dunnett's *post hoc* test; *$p < 0.05$ and **$p < 0.001$ compared to the corresponding controls. (Continued on next page)

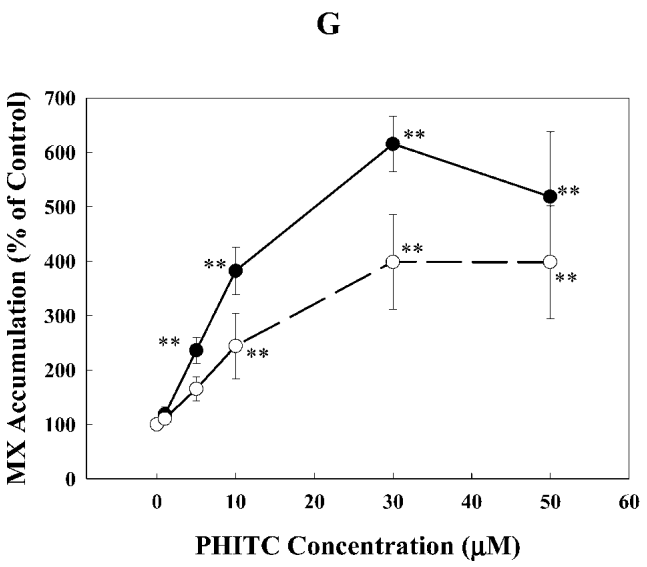

Fig. 2. Continued.

HITC ($p < 0.001$), and 5 μM of PHITC ($p < 0.001$) or PBITC ($p < 0.05$) significantly increased intracellular MX accumulation. No significantly greater increase in inhibition was observed at ITC concentrations of 50 μM in MCF-7/MX100 cells for NITC, HITC and PHITC, and IC_{50} values were around 10 μM (Fig. 2). Similarly, in NCI-H460/MX20 cells, all the tested ITCs significantly increased MX accumulation at a concentration of 30 μM ($p < 0.001$), and significant effects were also seen at 10 μM of PHITC ($p < 0.001$) or PBITC ($p < 0.001$), and 5 μM of NITC ($p < 0.001$) (Fig. 2). Except PEITC, all the other tested ITCs reached the maximal effect in NCI-H460/MX20 cells at 50 μM; and IC_{50} values were around 10 μM for BITC, PBITC, HITC and PPITC, and 5 μM for NITC and PHITC (Fig. 2). None of tested ITCs was effective at 1 μM. For the tested compounds, PHITC, NITC and PBITC were the most potent ITCs in inhibiting the BCRP-mediated efflux of MX.

Effect of ITCs on MX Cytotoxcity

MX cytotoxicity studies were conducted in order to confirm that the increased intracellular MX in BCRP-overexpressing cells produced by ITC exposure also resulted in improved cytotoxicity of MX in the presence of ITCs. NCI-H460/MX20 and NCI-H460 cells were treated by fixed doses of test compounds in combination with increasing concentrations of MX. In NCI-H460/MX20 cells, 10 μM of the tested ITCs generally inhibited cell growth significantly ($p < 0.001$), with IC_{50} values ranging from 9.48 to 23.3 μM of MX, lower than that of the cells treated only with MX (41.7 μM) (Table I). FTC, the positive control compound, reduced the IC_{50} of MX to 0.65 μM in NCI-H460/MX20 cells, whereas there was no significant effect by FTC in the parental cells (Table I). Consistent with the MX accumulation study, the most potent ITCs were PHITC, NITC and PBITC, 10 μM of which reduced IC_{50} values of MX in NCI-H460/MX20 cells by 4.4-, 2.5- and 3.8-fold, respectively (Table I). All the tested ITCs, when administered alone, did not inhibit the growth of NCI-H460 cells at the maximal tested concentrations, although NITC induced cell growth in NCI-H460 cells and increased the IC_{50} of MX by 1.8-fold (Table I). The IC_{50} values of NCI-H460/MX20 cells were more than 1000 times those of NCI-H460 cells and none of the tested compounds, including FTC, was able to reverse MX cytotoxicity completely back to the level of sensitive cells (Table I). Using ITC concentrations of 5, 10, and 20 μM, concentration-dependent effects were examined for NITC and PHITC, two of the most potent ITCs according to the MX accumulation studies, in NCI-H460/MX20 cells. As shown in Fig. 3, the higher the ITC concentrations, the more the cell growth curve shifted to the left, indicating increased sensitivity of the resistant cells to MX cytotoxicity. Both compounds significantly reduced IC_{50} values of MX to NCI-H460/MX20 cells at 5 μM ($p < 0.001$), 10 μM ($p < 0.001$) and 20 μM ($p < 0.001$), and the values were 20.1, 16.7 and 9.63 μM for NITC, respectively, and 14.3, 9.48 and 6.03 μM for PHITC, respectively (Table I). These trends

Table I. Effect of ITCs on MX Cytotoxicity in Parental and MX-Selected NCI-H460 Cells

ITC concentration (μM)	IC_{50} (nM) (NCI-H460) (Control 4.23 ± 1.78)		IC_{50} (μM) (NCI-H460/MX20) (Control 41.7 ± 12.4)		
	10	20	5	10	20
BITC	2.95 ± 0.75			19.8 ± 3.93[a]	
PEITC	2.21 ± 0.66			23.3 ± 9.54[a]	
NITC		7.96 ± 2.02[a]	20.1 ± 0.86[a]	16.7 ± 4.59[a]	9.63 ± 3.67[a]
PBITC	4.31 ± 1.12			11.0 ± 3.84[a]	
PPITC	3.83 ± 1.49			17.1 ± 8.99[a]	
HITC		2.08 ± 0.71			10.9 ± 1.73[a]
PHITC		2.80 ± 1.59	14.3 ± 2.73[a]	9.48 ± 2.70[a]	6.03 ± 1.35[a]
FTC	2.13 ± 0.72			0.65 ± 0.31[a]	

Cells were grown in 96-well plates for 2 days and incubated with MX of increasing concentrations of 0–300 or 0–1000 μM (for NCI-H460 or NCI-H460/MX20 cells, respectively) at 37°C in the absence or presence of specified concentrations of ITCs for 1 day, followed by incubation in fresh media for another day. FTC (10 μM) was used as a positive control. Cell growth was determined using a SRB assay. IC_{50} values were determined by fitting the cell growth curve as described in "Materials and Methods." The data are expressed as mean ± SD, n = 1 experiment performed in quadruplicate for NCI-H460 cells or n = 3 or 4 experiments performed in quadruplicate for NCI-H460/MX20 cells. Statistical analysis was conducted by a one-way ANOVA followed by Dunnett's *post hoc* test; [a]$p < 0.001$.

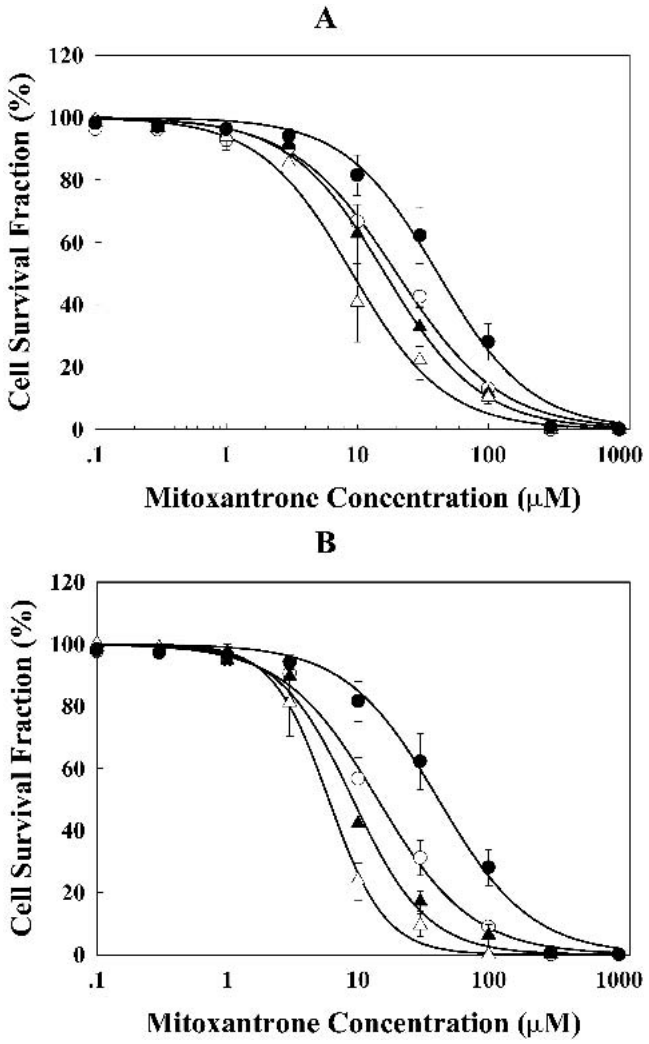

Fig. 3. The concentration-dependent effect of NITC and PHITC on the MX cytotoxicity in NCI-H460/MX20 cells. Cells were grown in 96-well plates for 2 days, and incubated with increasing concentrations of 0-1000 μM MX at 37°C in the absence (●) or presence of 5 (○), 10 (▲), and 20 (△) μM of NITC (A) or PHITC (B) for 1 day, followed by incubation in fresh media for another day. Cell growth was determined by a SRB assay and expressed as cell survival fraction, which was calculated as described in "Materials and Methods." The data are expressed as mean ± SD; n = 3 or 4 experiments, each performed in quadruplicate.

suggested that the ITC-mediated effects on MX cytotoxicity were dependent on ITC concentration.

Intracellular PEITC Accumulation in NCI-H460 and NCI-H460/MX20 Cells

To gain insight into the mechanism underlying the effect of ITCs on BCRP, intracellular accumulation of ^{14}C-PEITC was examined in NCI-H460 and NCI-H460/MX20 cells. Cells were treated by 5 μM PEITC with or without FTC or GF120918, the two BCRP inhibitors. As shown in Fig. 4, co-incubation of PEITC and 10 μM FTC or 2.5 μM GF120918 in NCI-H460/MX20 cells enhanced PEITC accumulation markedly by 7.4-fold ($p < 0.001$) or 3.7-fold ($p < 0.05$), respectively, indicating that the inhibition of BCRP resulted in more PEITC retained in the resistant cells. In comparison, the intracellular PEITC levels in NCI-H460 cells were unchanged in the presence or absence of either FTC or GF120918 (Fig. 4). In the presence of 5, 10, 25, 50, or 100 μM of PEITC, the intracellular levels of PEITC in NCI-H460/MX20 cells were all substantially lower than those in the corresponding NCI-H460 cells ($p < 0.001$), being only 11, 8, 17, 26, and 47% of the levels in the sensitive cells, respectively; addition of 10 μM FTC in NCI-H460/MX20 cells significantly improved PEITC accumulation by 6.8-, 4.6-, 4.1-, 2.7-, and 1.5-fold, respectively ($p < 0.001$) (Fig. 5). These results suggest that PEITC and/or its cellular metabolites were indeed transported by BCRP.

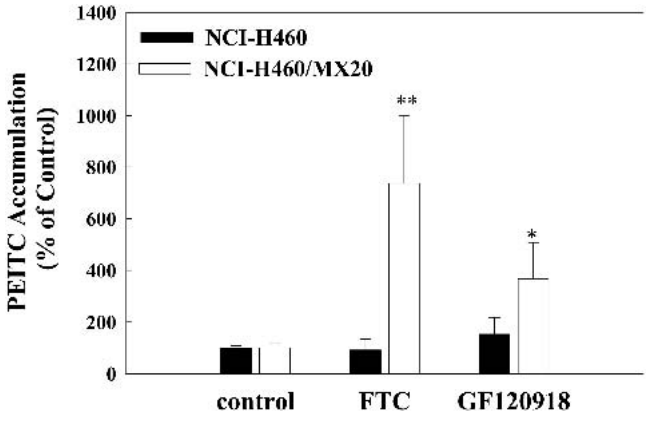

Fig. 4. PEITC accumulation in the parental NCI-H460 and MX-selected NCI-H460/MX20 cells. Cells were incubated with 5 μM ^{14}C-PEITC in the absence or presence of FTC (10 μM) or GF120918 (2.5 μM) in sodium buffer for 1 h. PEITC accumulation was normalized for cellular protein and presented as percent of control, where control represents cells treated with PEITC but without any modulator. Data are expressed as mean ± SD, n = 6; *$p < 0.05$ and **$p < 0.001$ by a one-way ANOVA followed by Dunnett's *post hoc* test.

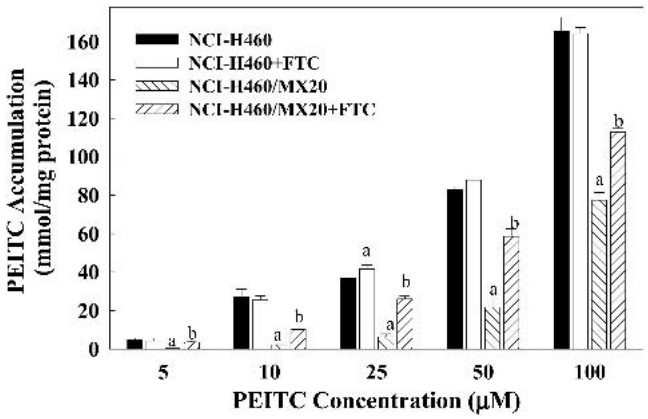

Fig. 5. Concentration-dependent PEITC accumulation in the parental NCI-H460 and MX-selected NCI-H460/MX20 cells. Cells were incubated with specified concentrations of ^{14}C-PEITC (supplemented with cold PEITC) in the absence or presence of FTC (10 μM) for 1 h. The intracellular PEITC accumulation was normalized by the amount of cellular protein. Data are expressed as mean ± SD, n = 6. Statistical analysis was conducted by a Student's *t* test: ([a]) significantly different compared to NCI-H460 cells treated with the same concentration of PEITC in the absence of FTC and $p < 0.001$ ($p < 0.05$ for the NCI-H460 cells treated with 25 μM PEITC in the presence of 10 μM FTC); ([b]) significantly different compared to NCI-H460/MX20 cells treated with the same concentration of PEITC in the absence of FTC and $p < 0.001$.

PEITC accumulation was not altered by the presence of FTC in NCI-H460 cells for all concentrations except 25 μM, where there was a small change ($p < 0.05$) (Fig. 5).

DISCUSSION

Like P-gp and MRP1, BCRP transports a variety of chemotherapeutic drugs resulting in MDR, a major reason for treatment failure in cancer therapy. Due to its high expression in normal tissues, BCRP also plays an important role in drug bioavailability, disposition and excretion. In the human jejunum, BCRP transcript is more abundant than MDR1 (for P-gp) with the rank order: BCRP ≃ MRP2 > MDR1 (12). The mRNA levels of ABC transporters are reported to correlate with the protein levels and BCRP showed a good correlation between transcript and protein levels in lung cancer cell lines (23,24). Therefore, the impact of BCRP in food-drug interactions may be important.

In this study, we examined the effect of ITCs, which represent a class of dietary compounds, on BCRP-mediated transport in both resistant and sensitive human breast cancer (MCF-7/MX100 and MCF-7/sensitive) and large cell lung carcinoma (NCI-H460/MX20 and NCI-H460) cells. Significant increases in MX accumulation were observed for BITC, HITC, PEITC, NITC, PHITC, PPITC and PBITC at concentrations of 50 μM in both MCF-7/MX100 and NCI-H460/MX20 cells, suggesting their inhibitory effect on BCRP. AITC, PITC, sulforaphane, erucin and erysolin did not affect MX accumulation in either of the BCRP-overexpressing cell lines. The increase of intracellular MX accumulation in MCF-7/MX100 cells was remarkable, ranging from 3.5- to 5.8-fold, and comparable to the positive control compound FTC (5-fold). Likewise, a similar trend was observed in NCI-H460/MX20 cells, although to a lower extent (2.4- to 3.1-fold) than in MCF-7/MX100 cells, and the intracellular MX accumulation between MCF-7/MX100 cells and NCI-H460/MX20 cells exhibited good correlation ($r^2 = 0.86$; $p < 0.01$). These findings suggest that the increased accumulation of MX caused by ITCs is BCRP related, and indicate that ITCs may be BCRP inhibitors. All the seven ITCs that increased MX accumulation in resistant cells produced small but significant effects in MCF-7/sensitive cells, and BITC, PEITC, and PPITC enhanced MX accumulation in NCI-H460 cells slightly but significantly as well. PITC, which did not show any effect in the resistant cells, enhanced MX accumulation in MCF-7/sensitive cells significantly. The underlying mechanisms for the increased MX accumulation in the parental cells are not known. Western blot analysis showed that neither of the parental cell lines had detectable BCRP, P-gp, or MRP1 expression (19), although this does not preclude the existence of low levels of these transporters in the cells. In fact, low expression of BCRP mRNA in NCI-H460 cells, as detected by Northern blot analysis, has been reported (25). However, FTC, a BCRP inhibitor with high potency, did not show any effect in the parental cells. Our laboratory previously investigated organic ITCs as inhibitors of P-gp- and MRP1-mediated drug resistance in cancer cell lines and demonstrated that BITC, PEITC and NITC can inhibit P-gp, while BITC, PEITC, NITC, PHITC, and PBITC inhibited MRP1. These ITCs were effective at concentrations of 50 μM, but had no effects at 10 μM (17,18). Therefore, ITCs only represent weak inhibitors of P-gp or MRP1; additionally, MX has been shown to be a weak P-gp substrate (26,27) but not a substrate for MRP1 (28). Therefore, it is possible that low expression of BCRP and/or P-gp in the parental cell lines may be responsible for the increased MX accumulation, or that there may be another transporter or mechanism involved in the ITC-induced increases in MX accumulation in the parental cell lines.

Concentration-dependent effects of the ITCs on MX accumulation were observed. The ITCs were effective at concentrations of 5 μM (NITC, PHITC, and PBITC), 10 μM (HITC) or 30 μM (BITC, PEITC, and PPITC). In both MCF-7/MX100 and NCI-H460/MX20 cells, PHITC, NITC, and PBITC appeared to be the most potent ITCs in reversing MX accumulation among the tested compounds. None of the ITCs exhibited significant effects at 1 μM. The ITCs generally showed higher activity in MCF-7/MX100 cells than in NCI-H460/MX20 cells as a result of the lower protein level of BCRP in NCI-H460/MX20 cells (19). However, NITC increased the cellular accumulation of MX at lower concentrations in NCI-H460/MX20 cells than in MCF-7/MX100 cells. The underlying mechanism is unknown, although the metabolism of NITC might be different from other ITCs in these two cell lines. The metabolism of NITC differs from that of the other ITCs, in that the glutathione conjugate that is formed is very labile (half-life of <1 min) (29), and the major metabolite formed *in vivo* is naphthylamine, formed predominantly by CYP1A1 (30). The results from MX cytotoxicity studies confirmed the MX accumulation study findings, in that ITCs with the most potent effects on MX accumulation had the most potent effects on the cytotoxicity of MX. On the whole, IC_{50} values of MX cytotoxicity were reduced significantly by 10 μM of ITCs in NCI-H460/MX20 cells. The ITCs did not increase MX cytotoxicity in the sensitive NCI-H460 cells at the maximum concentration used in the cytotoxicity studies, but 20 μM of NITC reduced MX cytotoxcity as represented by a higher IC_{50} value than control. In addition, the effects of ITCs on MX cytotoxicity are dependent on ITC concentrations. Taken together, the results of both accumulation and cytotoxicity studies demonstratated that ITCs are BCRP inhibitors. It appears that inhibitory activity is related to lipophilicity of the compounds, because the three most potent compounds, PHITC, PBITC, and NITC, are the most lipophilic, whereas those inactive (erucin, eysolin, sulforaphane, AITC and PITC) are the most hydrophilic compounds among the tested ITCs. On the other hand, ITCs with higher lipophilicity may exhibit a greater intracellular accumulation and effect, due to higher membrane permeability. All of the effective ITCs contained a phenyl ring except for HITC, but HITC has high lipophilicity. PITC, with a phenyl ring but a short backbone, did not inhibit BCRP at the tested concentration, suggesting both the backbone length and phenyl ring of ITCs may play a role in BCRP inhibition.

The concentration of ITCs that inhibit BCRP may be clinically relevant. It is estimated that milligrams of ITCs are consumed by humans per day through dietary vegetable sources (16). Some estimates of human consumption of glucosinolates, the ITC precursors, are as high as 300 mg/day (~660 μmol/d) (15). Recently, a number of food supplements containing ITCs or extracts of cruciferous vegetables have become commercially available. If 1.65 L is taken as the estimated gut volume (31) and 10 mg as the PEITC (~60 μmol) intake from the diet, the concentration of PEITC would be 37 μM. In a Phase I clinical study evaluating PEITC as a

chemopreventive agent, a single oral dose of 40 mg was administered to subjects (32). Using 1.65 L as the intestinal fluid volume, this amount will produce an intestinal concentration of 148 μM of PEITC. On the other hand, lumenal contents are mixed predominantly within smaller segments of the small intestine (33), suggesting local or segmental concentrations of a ingested food component in the intestine may be higher. Moreover, intracellular concentrations of ITCs are much higher than extracellular concentrations. For example, cells exposed to 100 μM concentrations of sulforaphane have intracellular concentrations of 6.4 mM (parent and GSH conjugate) (34). The minimal effective concentration of ITCs for BCRP inhibition ranged from 5 to 30 μM according to our study. The bioavailability of PEITC is high and clearance is low in rats (Ji Y and Morris ME, unpublished data), so little presystemic metabolism would be expected, in contrast to many other dietary components. As a result, ITC concentrations in the intestine may be high enough to affect BCRP-mediated transport.

PEITC is one of the most extensively studied ITCs because of its high potency against a variety of tumors and its low *in vivo* toxicity. Using PEITC as a model drug, we examined the intracellular accumulation of ^{14}C-PEITC in BCRP-overexpressing (NCI-H460/MX20) and BCRP-negative (NCI-H460) cells, in order to elucidate whether PEITC is a BCRP substrate. When the cells were incubated with different concentrations of PEITC ranging from 5 to 100 μM, the intracellular levels of PEITC in NCI-H460/MX20 cells were only 8–47% of those in the corresponding parental cells ($p < 0.001$). Co-administration of 10 μM FTC or 2.5 μM GF120918 enhanced ^{14}C-PEITC accumulation in NCI-H460/MX20 cells by 7.4-fold ($p < 0.001$) or 3.7-fold ($p < 0.05$), respectively; FTC or GF120918 did not alter PEITC accumulation in the parental NCI-H460 cells. Overall, these results suggest that PEITC and/or its cellular metabolites are BCRP substrates. Increasing the PEITC concentration resulted in a decrease in the percentage of intracellular PEITC concentration in NCI-H460/MX20 cells compared with that in NCI-H460 cells; the effect of FTC in increasing the accumulation of ^{14}C-PEITC was also decreased in NCI-H460/MX20 cells as PEITC concentration increased. Therefore, the BCRP-mediated transport of PEITC and/or metabolites appears to be saturable, supporting our hypothesis that PEITC is actively transported by BCRP. ITCs enter cells by passive diffusion and primarily form ITC-glutathione (-GSH) conjugates in cells. The intracellular accumulation of ITC-GSH is maximal after about 30 min in cultured cells (35). It was reported recently that AITC, BITC, and PEITC, were rapidly exported, mainly in the forms of GSH- and cysteinylglycine-conjugates, by P-gp and MRP1 (36). MRPs transport GSH-, glucuronide-, or sulfate-conjugates or cotransport substrates with GSH (37). BCRP is known to transport sulfate conjugates (8), but glutathione conjugates are poor substrates (38,39). Therefore, it is likely that PEITC, and not PEITC-GSH, is transported by BCRP; however, this has not been directly examined.

In conclusion, we have shown that organic ITCs reversed intracellular MX accumulation in BCRP-overexpressing MCF-7/MX100 and NCI-H460/MX20 cells, with small or no effect in BCRP-negative MCF-7/sensitive and NCI-H460 cells. ITCs exerted activity on intracellular MX accumulation in the resistant cells in a concentration-dependent manner, and significant effects were observed at ITC concentrations of 5, 10, or 30 μM. ITCs generally potentiated MX cytotoxicity to BCRP-overexpressing cells at concentrations of 5 or 10 μM but had no or small effects in BCRP-negative cells. Among the tested ITCs, PHITC, NITC and PBITC possessed the highest potencies. In addition, PEITC and/or its cellular metabolites may represent BCRP substrates. Our results demonstrate that organic ITCs inhibit BCRP-mediated transport and suggest the potential clinical significance of intestinal ITC-drug interactions.

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