JPP
Journal of
Pharmacy and Pharmacology

JPP 2003, 55: 1251–1257
© 2003 The Authors
Received March 26, 2003
Accepted May 28, 2003
DOI 10.1211/0022357021666
ISSN 0022-3573

Quantitative evaluation of isothiocyanates as substrates and inhibitors of P-glycoprotein

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Abstract

The ATP-binding cassette transporter P-glycoprotein (P-gp) exerts a critical role in the systemic disposition of, and exposure to, lipophilic and amphipathic drugs, carcinogens, toxins and other xenobiotics. The ability of P-gp to transfer a wide variety of structurally unrelated compounds from the cell interior across the membrane bilayer remains intriguing. Since dietary chemicals in cruciferous and several other foods appear to exert anticarcinogenic effects by inducing phase II enzymes and inhibiting some phase I enzymes, the isothiocyanate constituents are frequently studied for interactions with various biomacromolecules as well as cytotoxins or isolated cells. Several prominent dietary isothiocyanates were characterized for their interaction with P-gp and their specific effects on the P-gp export activity of several marker substrates. Some of these compounds inhibit the active P-gp-mediated efflux of the fluorescent markers LDS-751 and daunorubicin with low potency, with the most potent among them, phenethyl isothiocyanate, inhibiting transport of the LDS-751 substrate with an IC_{50} of $\sim$240 μM. Overall, these isothiocyanates are unlikely to impede the xenobiotic defence function of P-gp even in the intestine where the concentrations are potentially high.

Introduction

Transmembrane transport proteins are critical to the disposition of many xenobiotics. Cellular and tissue exposure are often dependent on the action of ATP-binding cassette (ABC) transporters, in particular P-glycoprotein (P-gp) (Gottesman & Pastan 1993; Gottesman et al 1996; Ambudkar et al 1999). Among ABC transporters, P-gp appears to have the broadest substrate acceptance (recognition) and perhaps the widest tissue distribution. Consequently, it is also the most intensely studied of the energy-dependent multidrug resistance (MDR) enzymes. This transport enzyme actively effluxes or ejects many structurally unrelated drugs from various tissues and organs, including the liver, intestine, brain, kidney and lymphocytes. Accordingly, P-gp has a significant effect on the bioavailability and distribution, and hence efficacy, of many drugs, including chemotherapeutic drugs, natural products, toxicants and peptides.

P-gp is a 170-kDa phosphorylated glycoprotein encoded by the MDR1 gene. It has two homologous halves, each of which contains a transmembrane domain(s) and an ATP-binding domain. This transport enzyme uses ATP (via hydrolysis) as the source of energy for "translocating" various substrates (Sharom et al 1993). An allosteric linkage has been shown between catalysis at one ATP-binding site and catalysis at the other homologous site (Senior & Bhagat 1998). Substrates enter from the lipid bilayer and bind to two (or more) non-identical sites (Shapiro & Ling 1997a; Sharom et al 1999) at the cytoplasmic leaflet (Shapiro & Ling 1997b). Allosteric and perhaps synergistic effects have been indicated for certain substrate combinations and conditions (Shapiro & Ling 1997a). Distinct but overlapping specificities of the drug binding/transport sites may help explain the broad substrate tolerance or lack of specificity of P-gp.

Some allosteric linkage between drug binding/transport sites also appears to exist. Although each of the two homologous halves of P-gp contains an ATP-binding site and perhaps a substrate-binding site, recent evidence indicates they are unequal yet linked. Kinetic data indicating non-competitive inhibition of P-gp-mediated transport

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Acknowledgements: We are very grateful to Professor Adriane L. Stewart for editorial assistance and to Sylvia Johnson for her comments.

by substrate combinations (or inhibitors) suggests differing substrate specificity between at least two binding sites (Litman et al 1997; Pascaud et al 1998; Wang et al 2000a, 2001a). Many of these studies show V_{max} changes that are consistent with non-exclusive binding of an inhibitor or alternative substrate, and photoaffinity labelling has indicated non-identical substrate-binding sites (Dey et al 1997). Moreover, recent evidence even suggests interaction between the two binding sites (Pascaud et al 1998; Wang et al 1998, 2000a) and/or the nucleotide-binding sites (Senior & Bhagat 1998; Hrycyna et al 1999; Loo & Clarke 1995). Such an allosteric linkage between sites can affect the characteristics of inhibition of marker substrate transport out of the cell by other competing substrates. Under these circumstances, the inhibition of transport may differ from that observed by simple hyperbolic saturation plot of inhibition (Wang et al 2000b).

Identification and characterization of specific dietary chemicals, and an increased understanding of their biological activity, elevate the distinction of natural product chemicals (phytochemicals) in disease prevention and treatment. Isothiocyanates are hydrolysis products from naturally occurring glucosinolates in a variety of foods of plant origin including broccoli, cabbage, Brussels sprouts, cauliflower, radish and watercress (Talalay et al 2001), as well as spinach, kale, collard greens, pak choi and kohlrabi. The flourishing interest in naturally occurring isothiocyanates as therapeutic agents is owing to credible evidence of the anticarcinogenic and antioxidant activities of these compounds, seemingly due to selective inhibition of some cytochrome P450 (CYP) enzymes and elevation of some phase II enzymes (Smith & Yang 2000; Talalay & Fahey 2001). CYP enzymes can activate some compounds to the reactive carcinogen, and many phase II conjugation enzymes catalyse the covalent adduction with small hydrophilic compounds, such as the tripeptide glutathione, to reactive compounds. Since these isothiocyanates can inhibit some CYP enzymes, it is reasonable to question whether they interact with P-gp (Wang et al 2001b). In this report, we have shown that a major isothiocyanate constituent of brassica vegetables, phenethyl isothiocyanate (PEITC), does not significantly impede the xenobiotic defence function of P-gp until high concentrations relative to expected intestinal quantities are administered.

Materials and Methods

Chemicals

Daunorubicin, verapamil, Na-orthovanadate, dithiothreitol, PEITC, benzyl isothiocyanate, ouabain, sodium azide, Mg-ATP, sodium dodecyl sulfate, ascorbic acid and ammonium molybdate were purchased from Sigma Chemical Co. (St Louis, MO, USA). 3-Morpholinopropanesulfonic acid was purchased from Acros (Morris Plains, NJ, USA). Phenylbutyl isothiocyanate and phenylpropyl isothiocyanate were purchased from LKT Laboratories, Inc. (St Paul, MN, USA). LDS-751 was purchased from Molecular Probes (Eugene, OR, USA). Ciclosporin was purchased from Alexis Biochemicals (San Diego, CA, USA). Hanks' Balanced Salt Solution, Alpha minimum essential medium, Dulbecco's modified Eagle's medium, penicillin/streptomycin, fetal bovine serum, and trypsin-EDTA were obtained from Life Technologies, Inc. (Rockville, MD, USA). All other reagents were of the highest grade commercially available.

Cell lines

The NIH-3T3-G185 cell line presenting the gene product of human MDR1 was licensed from NIH and maintained in Dulbecco's modified Eagle's medium as described previously (Wang et al 2000c).

FACS flow cytometry

Fluorescence measurements of individual cells were performed using a Becton-Dickinson FACScalibur fluorescence-activated cell sorter (San Jose, CA, USA), equipped with an ultraviolet argon laser (excitation at 488 nm, emission at 530/30 and 650/30 nm band-pass filters). Analysis was gated to include single viable cells on the basis of forward and side light-scatter and was based on acquisition of data from 10 000 cells. Log fluorescence was collected and displayed as single-parameter histograms. A modified inhibition assay for the P-gp efflux pump in viable transfected cells was performed with the flow cytometer as previously described (Wang et al 2000c). The efflux incubation was 15 min at 37 °C, and the marker substrate concentration of LDS-751 was 0.2 μM; daunorubicin was used at 2 μM.

Cell viability test

Cell viability was assessed using propidium iodide staining. Dead cells in which propidium iodide was bound to double strands of DNA or RNA were detected in certain regions of the cytometry dot plots and not included in the final data calculations.

Calculation of relative fluorescence

The fluorescence intensity of individual cells was recorded as histograms. The mean fluorescence intensity of 10 000 cells was used for comparison between different conditions. Ciclosporin or verapamil was selected as a positive control because it can significantly inhibit the P-gp-mediated active efflux of fluorescent substrate markers. Relative fluorescence was used for quantitation and comparison between different compounds.

ATP hydrolysis and phosphate release

The consumption of ATP was quantified by determining the amount of liberated inorganic orthophosphate, which forms a colour complex with molybdate. The ATP hydrolysis assay based on phosphate-release determination using membrane microsome preparations (human P-gp; Gentest, Woburn, MA, USA) was carried out in a 96-well

microplate (Sarkadi et al 1992). The microsomes were thawed at ~37 °C in a water bath and then placed on ice before diluting to 0.6 mg mL^{-1} (15 μg protein per well) in ice-cold ATPase buffer (KCl, 50 mM; dithiothreitol, 2 mM; 3-morpholinopropanesulfonic acid–Tris buffer, 50 mm; pH 7.0) containing 0.1 mM EGTA–Tris (to inhibit Ca-ATPase), 1 mM ouabain (to inhibit the Na/K-ATPase), and 5 mM sodium azide (to inhibit the mitochondrial ATPase). The total incubation volume including the various inhibitors was 51 μL. After pre-warming the above solutions to 37 °C, the incubation reaction was initiated by adding 16 mM Mg-ATP. The plate was then incubated for 20 min at 37 °C. The reaction was terminated by the addition of 40 μL 5% sodium dodecyl sulfate solution at room temperature. This was followed by the addition of 200 μL of a 8.75 mM ammonium molybdate/7.5% ascorbic acid (pH ~5.0) solution. The colour reaction was allowed to develop for 20 min at 37 °C, and the released phosphate was quantified by absorbance using a microplate reader (Tecan SpectraFluor Plus; RTP, NC, USA) at 830 nm. By comparison with a standard curve, the amount of phosphate released (and hence ATP hydrolysed) was quantified. The results were plotted as activity versus substrate concentration using GraphPad Prism software version 3.00 for Windows (San Diego, CA, USA).

Results

As fluorescent substrates transported by mammalian P-gp, daunorubicin, LDS-751, rhodamine 123 and others serve as markers for active transport function simply by measurement of retained fluorescence per cell (Wang et al 2001a). The IC_{50} (concentration at half-maximum inhibition) can be determined from a simple function, as shown in Figure 1, where the retained fluorescence was measured for samples of viable cells by a flow cytometer at varying concentrations of compound (Wang et al 2000c). The concentration dependency of inhibition displayed a sigmoidal response curve (Figure 1), a consequence of cooperativity (Wang et al 2000b), with the Hill equation for allosteric interaction enzymes therefore being the appropriate function for fitting to the data: $I = I_{max}S^n/(IC_{50} + S^n)$. The IC_{50} for PEITC against daunorubicin active efflux was approximately 380 μM in the NIH-3T3-G185 cell line (which over-expresses the gene product of human MDR1), and PEITC achieved about 1300% of relative control retention (Figure 1). It is clear from the control data in the figures that efflux inhibition effects on the parent (non-transfected) cell line are insignificant and therefore not contributing to the results.

Many of the isothiocyanates were also quantitatively characterized for their interactions with P-gp. None of these isothiocyanates exhibited the ability to significantly (potently) inhibit the P-gp-mediated active efflux of the substrate marker. PEITC also inhibits the efflux of LDS-751 (IC_{50} ~240 μM). Phenylbutyl isothiocyanate, phenylpropyl isothiocyanate and benzyl isothiocyanate inhibited the efflux of daunorubicin with similarly low potency (IC_{50} ~427, 338 and 400 μM, respectively) as well as LDS-751 (IC_{50} ~265, 304 and 168 μM, respectively).

With the observation that various isothiocyanates were able to affect the ability of P-gp to transport some fluorescent P-gp substrates out of a viable cell, we characterized several of these for transport kinetic parameters. As ATP is consumed at a purported rate of about one or two per transport event, the rate of hydrolysis of ATP represents transport rate or activity assay of function (Eytan et al 1996; Ambudkar et al 1997; Stein 1997; Shapiro & Ling 1998; Sauna & Ambudkar 2000, 2001; Wang et al 2000a). In the absence of exogenous substrate, the enzyme is still able to hydrolyse ATP to produce a basal level of activity, a reaction that is probably due to the transport of endogenous substrates. Therefore, activity data are presented as a percentage of the basal or control activity, as any change in the rate of ATP hydrolysis represents the sum of the basal activity and the contribution of the exogenous substrate to ATP hydrolysis. The presence of PEITC causes a concentration-dependent decrease in the rate of ATP hydrolysis relative to the baseline rate (Figure 2), which indicates that it is a comparatively slower substrate for P-gp than the apparent endogenous constituent causing baseline activity. P-gp commonly exhibits a basal activity that is purported to be caused by endogenous substrates (Shapiro & Ling 1998) such as membrane lipids that may be unfavourable to the cell interior or inner leaflet of the membrane (van Helvoort et al 1996). Not all P-gp transport substrates increase the rate of ATP hydrolysis; some substrates, such as PSC833 and gramicidin (Borgnia et al 1996), cause a decrease in the baseline ATPase activity with a hyperbolic relationship to concentration. As this response is consistent with a rate of transport that is even slower than that of putative endogenous substrates, this reaction pathway supplants the basal activity at sufficient concentrations by apparently competing with an endogenous substrate in the lipid milieu and being transported at a slower rate (Ramachandra et al 1996; Kerr et al 2001). The K_m is ~1 μM, and the V_{max} is ~74% of baseline, as indicated in Figure 2. The other isothiocyanates similarly reached activity at V_{max} that was lower than baseline ATP hydrolysis, although with relatively low K_m.

Discussion

Since P-gp is well characterized for a xenobiotic defence role, its chronic and effective inhibition in-vivo would not be generally beneficial to toxin response and protection. Indeed, absence of an interaction with P-gp at physiologically relevant concentrations should generally be consistent with chemoprotective qualities of plant-derived compounds, especially the isothiocyanates, whose highly touted anticarcinogenic effects have garnered much investigation as they are readily available in significant concentrations from cruciferous vegetables. Typical fractions of naturally occurring glucosinolate precursors in food plants such as cabbage, Brussels sprouts, cauliflower, turnip, radish and watercress range from about 0.5 to 3 mg g^{-1}

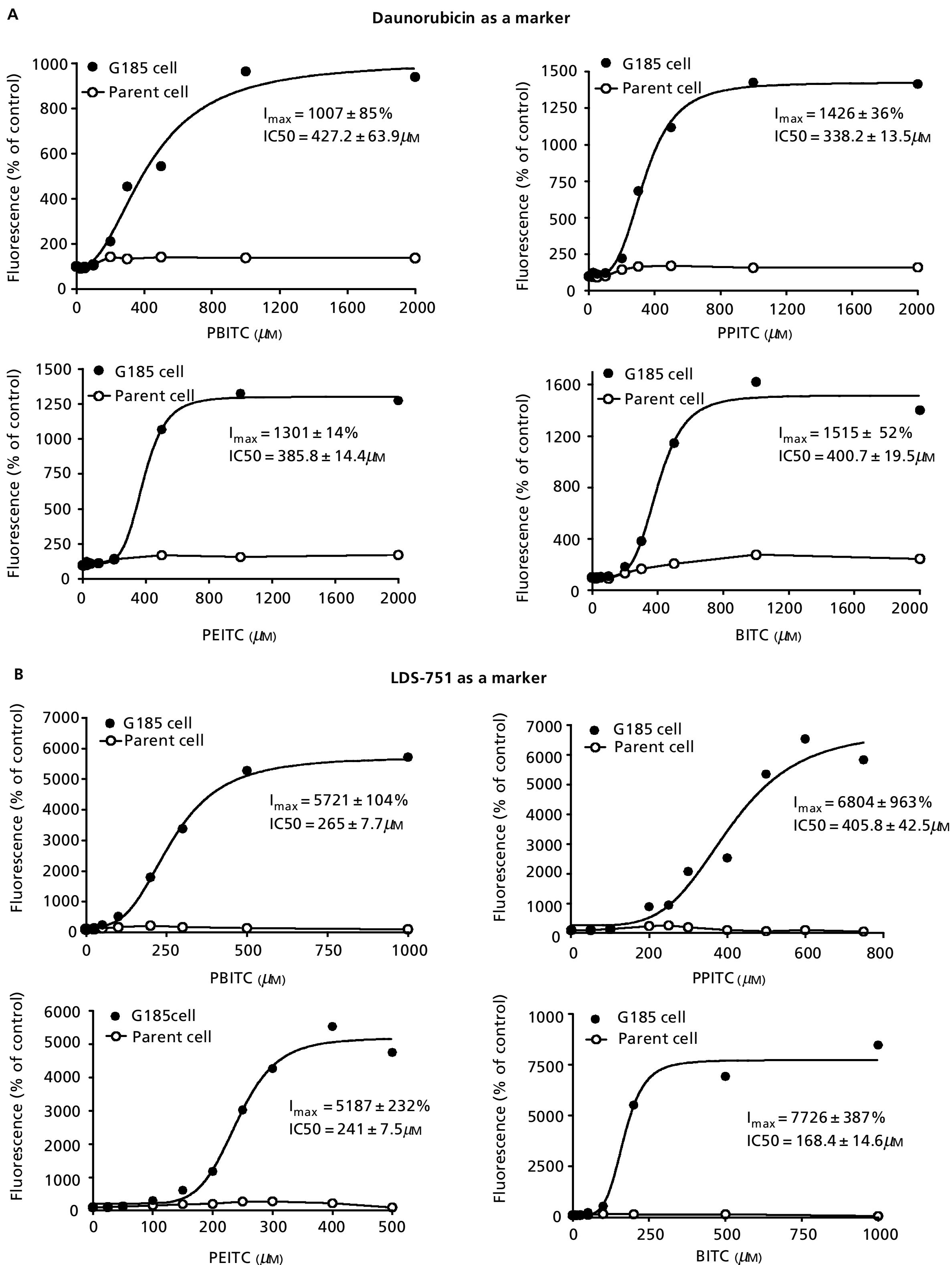

Figure 1 Intracellular retention of (A) daunorubicin or (B) LDS-751 in NIH3T3-G185 cells (•) and wild-type cells (○) versus competing isothiocyanate concentration. Fluorescence intensity is expressed as relative fluorescence. The average number of cells per assay was 10 000. The function for the line through the data is the Hill equation: $I = I_{max}S^n/(IC_{50} + S^n)$. The parameters IC_{50} and I_{max} (maximum inhibition) along with the standard deviation are shown. The isothiocyanates have no effect on retention of the marker substrate in the control cells (parent, non-transfected cell line). PBITC, phenylbutyl isothiocyanate; PPITC, phenylpropyl isothiocyanate; PEITC, phenethyl isothiocyanate; BITC, benzyl isothiocyanate.

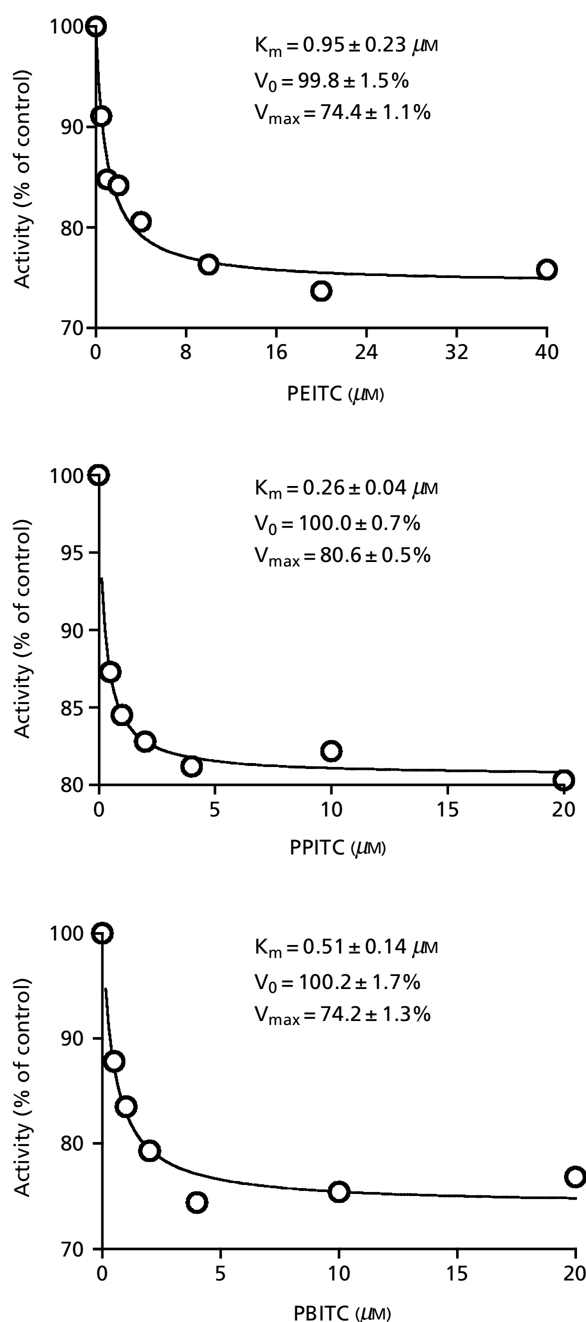

Figure 2 P-glycoprotein-mediated ATP hydrolysis rates in the presence of phenethyl isothiocyanate (PEITC), phenylpropyl isothiocyanate (PPITC) and phenylbutyl isothiocyanate (PBITC). Graphs are fit to a Michaelis–Menten saturation equation using non-linear regression analysis.

(Tookey et al 1980). PEITC uptake, for example, has been estimated at about 6 mg per ounce of watercress consumed (Chung et al 1992; Hecht et al 1995). Isothiocyanates at high doses absorb rapidly to maximum concentrations of 1–2 µM in plasma (Ye et al 2002); although upper intestine concentrations may conceivably achieve 20–23 µM, PEITC has an IC_{50} for P-gp that is roughly 10-fold above this.

The two primary mechanisms of anticarcinogenic action of the isothiocyanates appear to be the inhibition of carcinogen activation by inhibition of CYP enzymes and the elevation of detoxication by inducing phase II enzymes (such as glutathione *S*-transferase) (Smith & Yang 2000). Although generally facilitating the removal of toxins by oxidation, CYP enzymes have been shown to activate many pre-carcinogens to their reactive adduct-forming metabolites, with the inhibition of this process also able to mitigate specific DNA and protein damage. Phase II enzymes such as glutathione *S*-transferases catalyse the addition of the hydrophilic tri-peptide glutathione to electrophilic centres of reactive toxins, thereby providing a chemoprotective or xenobiotic defence role. As P-gp often contributes a significant role in detoxication as well, it would usually be confounding to inhibit P-gp too. Moreover, the recent heightened attention to drug–herb interactions caused by herbal remedies suggests it would be preferential (to chemoprotection) that isothiocyanates not inhibit P-gp in addition to the CYP enzymes. Indeed, it should be counter to chemoprotection to inhibit the ability of P-gp to remove cytotoxins.

We have clearly shown an absence of significant inhibition of P-gp-mediated transport of two very well characterized and comprehensive substrate markers of transport activity. Although some interaction has been reported, it was limited to PEITC at 100 µM (Tseng et al 2002). The potency of interaction is so low as to be physiologically irrelevant, even at the potentially high doses of isothiocyanates expected in the gastrointestinal tract with some cruciferous vegetables. Given the recent increased attention to the pharmacokinetic interactions between herbal remedies and medicinal drugs (Ioannides 2002), it is important to address the potential inhibition of P-gp (a major mediator of drug interactions). Indeed, the isothiocyanates are responsible for interactions with drugs via the inhibition of some CYP enzymes. However, there are known differences between lists of CYP inhibitors and P-gp inhibitors (Wang et al 2001b).

The second considered physiological response and mechanism of interaction is the induction of xenobiotic defence enzymes. Isothiocyanate consumption has been shown to moderately induce glutathione *S*-transferase, UDP-glucuronosyltransferase, NADPH-quinone oxidoreductase and antioxidant enzymes, all of which could enhance the elimination of carcinogens. Significantly, the induction or enhanced activity of P-gp could be exceptionally chemoprotective due to its dramatic, well-characterized role in xenobiotic defence. Therefore, elevation of P-gp quantity by an isothiocyanate may be chemoprotective depending on cellular exposure and the particular carcinogen substrate. Since this increased P-gp activity could be a mechanism of apparent carcinogenesis mitigation resulting from brassica vegetable consumption, it deserves further investigation using in-vivo experiments.

There is the possibility of interaction with other ABC transporters such as multidrug resistance-associated proteins 1 and 2. These (and some other) transporters recognize glutathione conjugates and cells take up isothiocyanates by way of glutathione conjugation (Zhang 2001). It is not surprising,

therefore, that efflux of some of these isothiocyanate–glutathione conjugates is mediated by multidrug resistance-associated proteins (Zhang & Callaway 2002). Any potential interaction with multidrug resistance-associated proteins would require a very high affinity with the conjugate due to the already low concentrations of the isothiocyanates.

In conclusion, major vegetable component isothiocyanates do not inhibit active efflux via P-gp with significant potency (at low concentrations). Therefore, under typical dietary circumstances, these foodstuff compounds should not interact with the xenobiotic defence function of P-gp or with other drugs.

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