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PRINCIPAL INVESTIGATOR: Marilyn E. Morris, Ph.D.

CONTRACTING ORGANIZATION: State University of New York at Buffalo
Amherst, New York 14228

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Marilyn E. Morris, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
State University of New York at Buffalo
Amherst, New York 14228

**E-Mail:** memorris@acsu.buffalo.edu

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**13. ABSTRACT (Maximum 200 Words)**

Drug resistance is the main cause for therapeutic failure and death in breast cancer. Our goal is to evaluate dietary organic isothiocyanates (ITCs) as inhibitors of MDR. Our studies have demonstrated that phenethyl ITC (PEITC), benzyl ITC (BITC) and naphthyl ITC (NITC) can inhibit P-glycoprotein-mediated efflux in cell lines that overexpress P-gp, as well as in cell lines that overexpress another MDR protein, Multidrug Resistance-associated protein (MRP1). Studies evaluating the mechanism of this interaction have suggested that PEITC is an inhibitor, but not a substrate for P-gp. ITCs inhibit MRP1 through binding interactions, as well as the depletion of the cofactor for transport, glutathione. HPLC assays have been developed to determine the concentrations of these ITCs in biological samples, and a novel LC/MS/MS assay developed for PEITC, in order to obtain the needed specificity and sensitivity for in vivo studies. The stability and pharmacokinetics of NITC and PEITC have been determined. Both NITC and PEITC exhibit dose-dependent disposition, with clearance decreasing with increasing dose. The bioavailability of PEITC was determined for the first time, and found to be excellent (>80%). The ITCs may represent a new class of inhibitors of MDR in breast cancer.

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**Annual Research Report June 1, 2002-May 31, 2003**
**Organic Isothiocyanates: Dietary Modulators of Doxorubicin Resistance in Breast Cancer**
**P.I.: Marilyn E. Morris, Ph.D.**

## INTRODUCTION

Drug resistance is the main cause for therapeutic failure and death in breast cancer. An important mechanism of this resistance is the enhanced cellular efflux of a wide variety of structurally distinct classes of chemotherapeutic agents due to the overexpression of *P-glycoprotein (P-gp)*. In a recent study, Buser et al. (1997) reported a high prevalence of *P-gp* in breast cancer tumor tissue: 83% in early breast cancer and 100% in primarily metastatic breast cancer. One strategy for reversing *P-gp*-mediated multidrug resistance (MDR) in breast cancer has been the concomitant use of chemical agents that are by themselves nontoxic but that potentiate the accumulation of chemotherapeutic drugs in MDR cells. Current attempts to reverse MDR with inhibitors have been largely unsuccessful due to the dose-limiting cytotoxicity of the inhibitors, and due to toxicity produced as a result of the altered pharmacokinetics of the chemotherapeutic agents. We propose the use of a new class of drugs, the organic isothiocyanates (ITCs), as inhibitors of *P-gp* -mediated doxorubicin (DOX) resistance in breast cancer. The organic ITCs are components present in the diet, especially in cruciferous vegetables such as broccoli, watercress, cabbage and brussel sprouts. These compounds are of considerable interest since they have chemoprotective properties; they are potent inhibitors of enzymes involved in carcinogen activation and inducers of enzymes involved in carcinogen detoxification. We have found that three organic ITCs, phenethyl, benzyl and naphthyl ITCs, can increase the accumulation of daunomycin in the drug-resistant human breast cancer cell line MCF-7, without affecting accumulation in sensitive MCF-7 cells. We are particularly interested in these compounds as *P-gp* inhibitors first because of their chemoprotective properties and secondly because they have been shown to be nontoxic in all studies to date. Our hypothesis is that these dietary ITCs, by inhibiting *P-gp* will reverse the tumor resistance to DOX, resulting in increased efficacy in breast cancer treatment, without increasing toxicity. In the proposed research, we would like to characterize the disposition of these ITCs in animal studies, determine their concentration-dependent effects on DOX disposition, and determine their effects on the efficacy and toxicity of DOX in a murine breast cancer model. Both the free and liposomal dosage forms of DOX will be examined since there is evidence that combining a resistance modifier with a liposomal form of DOX provides increased efficacy without altering DOX pharmacokinetics. The proposed research will represent the first investigation of the effect of this new class of dietary *P-gp* inhibitors on DOX resistance in breast cancer and will evaluate the effects of ITCs on both the free drug and liposomal drug dosage forms. Additionally, these studies will provide information on the extent of absorption and disposition of unchanged ITCs in the blood, information that is not currently available. This information is essential for the use of these compounds either as chemopreventive or chemosensitizing agents in breast cancer therapy.

## BODY

This represents the third annual report for this grant, since a one year no-cost extension was granted. The no-cost extension was requested for the following reasons:

1. My laboratory had a slow start due to a change in technical personnel in the first year of the study. My technician, Pat Neubauer left the laboratory and there was a considerable lag in time hiring a suitable replacement and training the person.
2. We had difficulties in developing analytical procedures with the sensitivity and specificity to assay phenethyl isothiocyanate (PEITC) in biological samples.
3. We spent a considerable length of time determining the stability of all of the three isothiocyanates, but in particular naphthyl isothiocyanate (NITC), which proved to be quite unstable in biological samples.

### Statement of Work

**Task 1.** Determine the concentration-dependent effect of BITC, PEITC and NITC on the accumulation of $^{3}$H-daunomycin in sensitive and resistant MCF-7 and SK-BR-3 human breast cancer cells, and in the porcine kidney cell line LLC-PK$_1$. This task will provide an estimate of the free blood concentrations that may be necessary to inhibit *P-gp* in vivo.
Time: Year 1 (2-4 months)

**Overview:**
This task has been completed. Three organic isothiocyanates (NITC, PEITC, BITC) were found to have significant inhibitory effects on the 2-hour cellular accumulation of DNM and/or VBL in resistant MCF-7 cells or in LLC-PK1 cells. These compounds had no effect on DNM accumulation in sensitive MCF-7 cells. PHITC increased the accumulation of VBL in resistant MCF-7 cells, but also increased DNM accumulation in sensitive MCF-7 cells, suggesting that its effect was not mediated through inhibition of P-gp. This information, as well as studies examining the effect of PEITC, BITC and NITC on the cytotoxicity of doxorubicin were reported in the 2000-01 Annual Report. These results have been published (Tseng et al., Pharm Res 19:1509-1515, 2002). We have performed some additional studies to evaluate other cell lines to examine the mechanism of inhibition. We have demonstrated that PEITC does not appear to be a substrate for P-glycoprotein.

**Abstract.** The objective of this investigation was to evaluate the effects of organic isothiocyanates (ITCs) on P-glycoprotein- (P-gp) and multidrug-resistance protein 1- (MRP1) mediated efflux of anticancer drug daunomycin (DNM), determine whether ITCs are substrates of P-gp and/or MRP1, and elucidate mechanism(s) involved in the inhibition of transport. Two dietary ITCs benzyl- (BITC) and phenethyl isothiocyanate (PEITC), and one synthetic ITC α-naphthyl isothiocyanate (1-NITC) were studied for their effects on 2-h accumulation of DNM in human breast cancer MCF-7 (sensitive), MCF-7/ADR (P-gp overexpression), colonic adenocarcinoma Caco-2 cells (P-gp and MRP2 expression), and pancreatic adenocarcinoma PANC-1 cells (MRP1 overexpression). BITC, PEITC and 1-NITC significantly increased the accumulation of DNM in MCF-7/ADR, Caco-2 (except for 1-NITC), and PANC-1 cells, with P-gp and MRP1 inhibitors verapamil (VRP) and MK571, respectively, as the positive controls. Isocyanate and amine metabolites of PEITC, BITC and NITC had no effect on the uptake of

DNM, suggesting that these metabolites are not inhibitors (Figure 1). $^{14}$C-PEITC was used for substrate studies in human breast cancer MDA435/LCC6 (sensitive), MDA435/LCC6MDR1 (P-gp overexpression), Caco-2 and PANC-1 cells. The uptake of PEITC was not changed in Caco-2, MDA435/LCC6 and MDA435/LCC6MDR1 cells in the absence and presence of VRP (Figure 2), but dramatically increased in the presence of MK571 when PEITC was incubated at concentrations of 1, 5, and 20 μM in PANC-1 cells. After short (2 h) and longer term (24 h) drug treatment, cellular concentrations of reduced glutathione (GSH) in PANC-1 (2 and 24 h) and Caco-2 (2 h) cells were profoundly depleted by BITC and PEITC by 6-100-fold, in a concentration-dependent manner, but not by 1-NITC, suggesting the mechanism of BITC and PEITC may be different from that of 1-NITC. Cellular activities of glutathione-S-transferase (GST) in both cell lines were not found to be changed after treatment. The results indicate that PEITC and/or the glutathione conjugate of PEITC (PEITC-NAC) are substrates of MRP1 rather than P-gp. The increased accumulation of DNM by BITC and PEITC in MRP1-overexpressing cells is probably due to the depletion of cellular GSH concentration as a co-substrate in DNM efflux, and the competitive binding of the glutathione conjugates of BITC and PEITC to a substrate binding site on MRP1. The mechanism underlying the effects of 1-NITC are not known, but its lack of effect in Caco-2 cells is likely due to its rapid metabolism in this cell line.

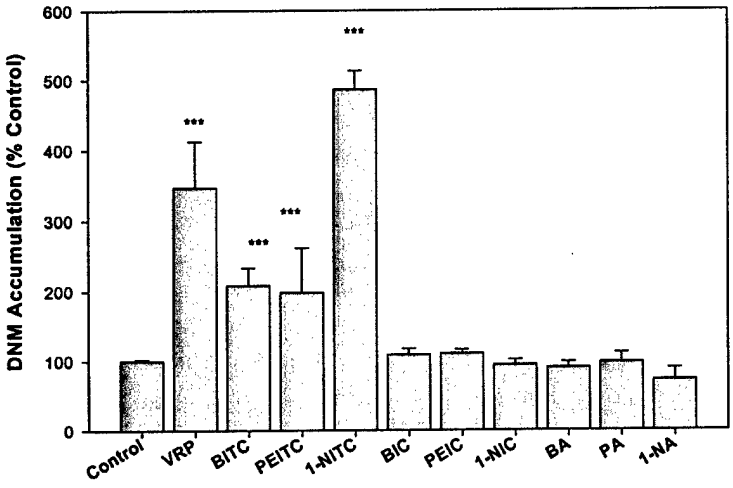


Figure 1. The effect of isothiocyanates, isocyanates and amines at 100 μM on accumulation of DNM (0.05 μM) in MCF-7/ADR cells (n = 6). The data are expressed as Mean ± SD, *** $P <$ 0.001.

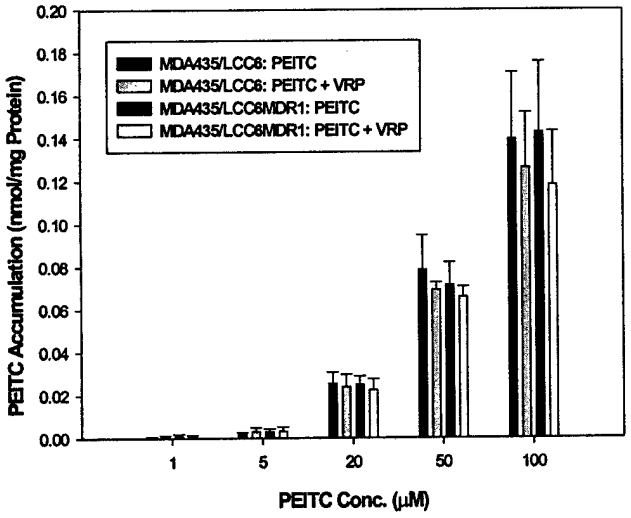


Figure 2. The effect of VRP (100 μM) on accumulation of PEITC in MDA435/LCC6 and MDA435/LCC6MDR1 cells (n = 6). The data are expressed as Mean ± SD.

**Task 2.** Synthesize $^{14}$C-labelled NITC, BITC and PEITC. The procedure for the synthesis of $^{14}$C-PEITC used by Conaway et al (1999) is well described and shown in Figure 3. We will begin with the synthesis of radiolabeled PEITC using this method.
Time: Year 1 - Year 2

**Overview**:
This task has been completed. $^{14}$C-PEITC was synthesized and purified; this was reported in the 2001-02 annual report.

**Task 3.** Set up HPLC assays for NITC, BITC and PEITC. Literature assays for BITC and PEITC have been described and will be set-up and optimized in the laboratory for the analysis of extracted blood, urine and bile samples. Stability of samples is a concern and will be addressed. Synthesize the mercapturic acid conjugates (the major metabolites) of PEITC and BITC.
Time: Year 1

**Overview**:
This task has been completed. We have developed HPLC assays for PEITC, BITC and NITC, as well as performing extensive stability studies for all three compounds. Since the major metabolites of PEITC and BITC are not commercially available, we synthesized and purified these conjugates, to use as standards for our analysis of biological samples. The HPLC assays for all 3 compounds have been previously reported in the 2000-01 and 2001-02 annual reports. We have published the assay and stability studies of 1-NITC this year (Hu K and Morris ME: Determination of 1-naphthylisothiocyanate (1-NITC) and metabolites 1-naphthylamine (1-NA) and 1-naphthylisocyanate (1-NIC) in rat plasma and urine by high-performance liquid chromatography, J Chromatography B 788:17-28,2003). The manuscript for the HPLC assays for PEITC and BITC is in preparation.

We have developed a novel LC/MS/MS assay for PEITC, which we have submitted for publication (Ji Y and Morris ME: Determination of Phenethyl Isothiocyanate in Human Plasma and Urine by Ammonia Derivatization and Liquid Chromatography-Tandem Mass Spectroscopy. Submitted 5/26/03). A description of the assay is given below:

**LC/MS/MS Assay for PEITC**

Phenethyl isothiocyanate (PEITC) is a dietary compound present in cruciferous vegetables that has cancer preventive properties. Our objective was to develop and validate a novel liquid chromatography-tandem mass spectrometry (LC/MS/MS) procedure to analyze PEITC concentrations in human plasma and urine. Following hexane extraction, ammonia was added to samples to derivatize PEITC to phenethylthiourea. Chromatographic separation was achieved on a $C_{18}$ column with acetonitrile/5 mM formic acid (60:40, v/v) as the mobile phase followed by tandem mass spectrometry detection in multiple reaction monitoring mode. Deuterium-labeled PEITC was used as the internal standard. The detection limit was 2 nM and calibration curves were linear from 7.8 to 2000 nM. The intra- and inter-day coefficients of variation were less than 5% and 10% respectively. The intra- and inter-day accuracy ranged from 101.0 to 104.2% and 102.8 to 118.6%, respectively. The recovery from spiked human plasma and urine ranged from 100.3 to 113.5% and 98.3 to 103.9%, respectively. The assay was used to measure PEITC

in plasma and urine samples obtained from subjects after consumption of 100 g of watercress. This novel assay represents the first analytical method with the sensitivity and specificity to determine plasma and urine concentrations of PEITC.

**Task 5.** Set up HPLC assay for doxorubicin and its metabolites.
Time: Year 2 (6-12 months)

**Overview**:
This task has been completed. A HPLC assay using fluorescence detection was set up in the laboratory for the analysis of doxorubicin. The analysis of DOX by high-performance liquid chromatography (HPLC) methods with fluorescence detection has been well documented in a variety of biological samples. An HPLC assay was set up and the lower limit of quantitation (LLQ) and linearity for the standard curve were determined and reported in the 2001-02 annual report.

During the year, our consultant's laboratory (Dr. Robert Straubinger, University at Buffalo) has developed an LC/MS/MS assay for doxorubicin and its metabolites. Although Dox and several metabolites are fluorescent, they are poorly resolved chromatographically and difficult to quantify using fluorescence. A rapid extraction method was developed in which the tissue samples were ground in liquid nitrogen, homogenized in mobile phase, clarified by centrifugation, and analyzed by LC-MS/MS. Samples (10uL) were introduced into a Perkin Elmer - Sciex API 3000 liquid chromatography tandem mass spectrometer (LC-MS/MS) via a turbo ion-spray source in positive ion mode. Separation was achieved under isocratic conditions using a reversed phase C18 guard and an analytical (4.6 x 50 mm 3.5 μm packing) column at a flow rate of 250 μL/min. An optimal mobile phase consisted of water:acetonitrile (60:40 v/v) containing 5 mM ammonium acetate (pH 3.5) provided sufficient separation from the void front and resulted in analysis times of 4 minutes. Assay performance (i.e., selectivity, sensitivity, linearity, and accuracy) was determined by injection of standards and quality control solutions prepared in blank plasma or tissues of interest. Plasma, liver, spleen, heart, lung, brain, and brain-tumors were harvested from Fisher 344 rats bearing 9L brain tumors after weekly administration of 5.67 mg/kg of free or Dox encapsulated in long circulating liposomes (SSL-Dox). Extraction efficiencies of 80-112% were achieved reproducibly for tissues examined. Instrumental parameters were optimized for parent and product ions for Dox and metabolites. The LC-MS/MS assay was linear over the range of therapeutically relevant plasma/tissues concentrations (0.247-1000 nM), with a lower limit of quantification of 0.247 nM and a sensitivity of ~2.8 pg achieved in brain tissue. Intra-day coefficients of variation for all tissues were less than 20%.

**Task 6.** Evaluate the effects of NITC, BITC and PEITC on the blood and urinary concentrations of unchanged DOX and its major metabolites following the i.v. administration of both the free and liposomal forms of DOX (7.5 mg/kg) in rats in vivo. These studies will evaluate whether these ITCs produce concentration-dependent changes in the metabolism, distribution and elimination of DOX when administered in either free or liposomal form. Again, we may limit our studies to two compounds at this point, based on our previous findings. (Three ITCs will be administered at 2 doses for 8 rats/group receiving either free or liposomal DOX. Approximate total number of rats is 100.)

Time: Year 2 (last 6 months) - Year 3 (first 3 months)

**Overview**: This task is in progress. In order to dose NITC and PEITC in rats, studies to determine the bioavailabity and disposition in rats were necessary. This information is not available, so these studies represent the first determination of the detailed pharmacokinetics of these compounds. Pharmacokinetic studies for NITC and PEITC were completed during 2002-03. Our progress has been somewhat slow in this area due to the time that was necessary to develop assay methods with the specificity and sensitivity needed to analyze plasma concentrations following relevant (low) doses. We also needed to address stability concerns during the drawing and processing of the biological samples.

**a) Pharmacokinetics of α-naphthylisothiocyanate (1-NITC) in rats.**

**Purpose:** To investigate pharmacokinetics of α-naphthylisothiocyanate (1-NITC) in rats.

**Methods:** Pharmacokinetic studies of 1-NITC were performed with four doses of 10, 25, 50 and 75 mg/kg to Sprague-Dawley female rats (n = 4 for each group; body weight 200-250 g) via i.v. administration. Blood samples (250 μl each) were collected from the jugular vein at 5, 10, 20, 30 min, 1, 2, 4, 6, 9, 12, 24, 36 and 48 h (36 and 48 h for 50 and 75 mg/kg groups). The concentrations of 1-NITC in plasma were determined by HPLC assay with C18 column (125 × 4.6 mm i.d., 5 μm), a mobile phase consisting of ACN-$H_2O$ (70:30, v/v), flow rate at 1.0 ml/min, and the detection wavelength at UV 305 nm. The data were simultaneously fitted using ADAPT II software.

**Results:** 1-NITC exhibited nonlinear Michaelis Menten disposition and data were characterized with a two compartment open model. Parameters were estimated as: maximium velocity ($V_{max}$), 2.13 ± 0.20 mg/h/kg; Michaelis Menten constant ($K_m$), 0.51 ± 0.13 mg/L; first order rate constant from central to tissue compartment ($k_{12}$), 1.10 ± 0.15 $h^{-1}$; first order rate constant from tissue to central compartment ($k_{21}$), 0.32 ± 0.04 $h^{-1}$; volume of central compartment ($V_C$), 3.37 ± 0.21 L/kg; volume of tissue compartment ($V_T$), 11.72 ± 0.85 L/kg.

**Conclusion:** 1-NITC demonstrated nonlinear pharmacokinetics via i.v. administration. These results will be used to support the application of 1-NITC in combination with anticancer drug doxorubicin (DOX) to reverse P-glycoprotein (P-gp)- and Multidrug Resistance Protein 1 (MRP1)-mediated multidrug-resistance (MDR).

**b) Pharmacokinetics of Phenethyl Isothiocyanate (PEITC) in Rats**

Studies are in progress to evaluate the pharmacokinetics and bioavailability of PEITC in rats. PEITC was dissolved in 15% (2-hydroxypropyl)-beta-cyclodextrin and administered intravenously or orally by gavage to rats. Various doses were administered. Plasma and urine samples were collected over time and PEITC concentrations analyzed by our LC/MS/MS assay. Pharmacokinetic parameters were obtained by fitting the plasma concentration-versus-time data to a two compartment model and by noncompartmental analysis, using the computer program WINNonlin (Pharsight Inc.).

PK parameters (from two-compartment analysis) following i.v. administration of PEITC:

a). Intravenous dose of 2 umol/kg BW PEITC (n = 3):

| | Average | sd | cv% |
|---|---|---|---|
| CL (L/h•kg) | 0.73 | 0.22 | 29.48 |
| $V_c$ (L/kg) | 0.72 | 0.11 | 15.23 |
| $CL_D$ (L/h•kg) | 0.94 | 0.19 | 20.08 |
| Vβ (L/kg) | 1.81 | 0.48 | 26.66 |
| β ($h^{-1}$) | 1.01 | 0.20 | 20.02 |

b). Intravenous dose of 20 umol/kg BW PEITC (n = 4):

| | Average | sd | cv% |
|---|---|---|---|
| CL (L/h•kg) | 0.46 | 0.15 | 33.32 |
| $V_c$ (L/kg) | 0.41 | 0.11 | 28.00 |
| $CL_D$ (L/h•kg) | 0.37 | 0.14 | 37.90 |
| Vβ (L/kg) | 0.80 | 0.05 | 6.23 |
| β ($h^{-1}$) | 1.17 | 0.43 | 36.39 |

Where CL= clearance; $V_c$=volume of the central compartment; $CL_D$ = distributional clearance; $V_\beta$= volume of distribution in the beta phase and β=terminal elimination rate constant.

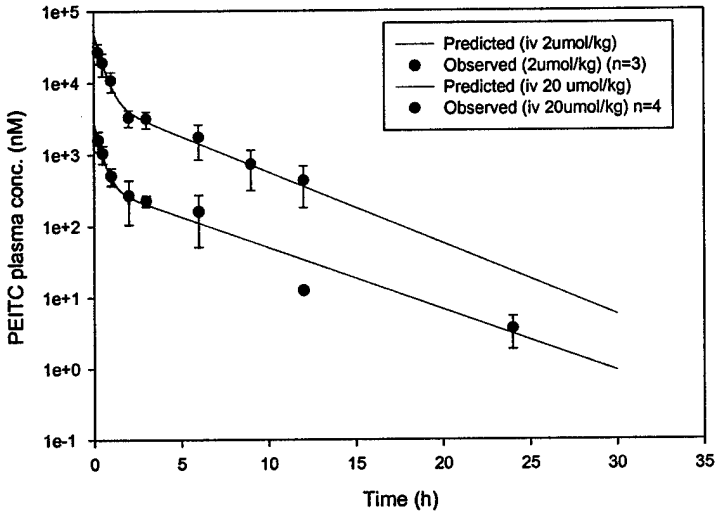


**Figure 3. PEITC plasma concentration versus time relationship after i.v. (2 μmol/kg) and oral (20 μmol/kg) administration to rats.**

Results and preliminary conclusions: PEITC demonstrates dose-dependent clearance with lower clearance after a higher dose. Bioavailability is excellent in rats, approaching 100%.

**Task 7.** Evaluate the effects of NITC, BITC and PEITC on the metabolism and biliary excretion of DOX in the perfused rat liver. (Three ITCs will be examined at 2-3 concentrations (examined in one rat liver preparation) with 8 rats/group receiving free or liposomal DOX. Approximately a total of 50 rats will be used.)
Time: Year 3 (first 3 months)

**Overview**: This task is in progress and will be completed in 2003-04. We are evaluating the effect of PEITC at a 3 μM concentration following a 4-day incubation period on the gene expression in human hepatocytes. We are using the GEArray® Q series Human Drug Metabolism Superarrys containing 96 genes encoding for drug metabolizing enzymes and transporters. Briefly, cDNA probes are synthesized by reverse transcription using 1 μg of the control (vehicle treatment) or treated RNA samples as the templates and labeled with $\alpha$-$^{32}$P-dCTP (10 μCi/μl; 3000 Ci/mmol, Amershan Pharmacia BioTech, Piscataway, NJ). The cDNA probes are then denatured and hybridized with GEArray® membranes. The hybridization signal was detected with a phosphor imager (Packard Instruments, Meriden, CT) and the relative abundance of a particular transcript was normalized against the signal of β-actin. The differences between the control and treated RNA samples will be evaluated significant analysis of microarrays (SAM) and student's t-test. We have decided to initially use cDNA arrays in order to examine a wide range of metabolizing enzymes and transporters, then to confirm our results using both in vitro activity studies and in vivo studies.

**Task 8.** Set-up a murine animal model of breast cancer through the s.c. implantation of both resistant and sensitive MCF-7 cells. Methods will be set up to determine antitumor effect and toxicity. (Approximately 20 mice will be used.)
Time: Year 3 (3-6 months)

**Overview:** This task will begin during the summer of 2003. We will begin setting up our animal model this summer with Dr. Atif Awad (Nutrition Program, University at Buffalo), who has considerable experience with xenograph murine cancer models (1,2).

**Task 9.** Evaluate the effect of NITC, PEITC and BITC on the efficacy and toxicity of DOX, administered in both free and liposomal forms at a dose of 6-10 mg/kg in the murine breast cancer model. Only two of these ITCs will be used in these studies, depending on the results of previous studies. Efficacy will be evaluated by examining (1) tumor volume and growth delay, (2) fraction of surviving tumor cells and toxicity will be assesssed by determining weight loss, hematologic status (leukocyte, erythrocyte and platelet counts in blood) and cardiac effects (as determined by tissue histology). (Eight groups of mice, as described in the Methods section, with 10 mice/group, treated with 2 ITCs in separate studies. Mice with both sensitive and resistant MCF-7 xenografts will be used. Approximate total number of mice is 320.)
Time: Year 3: last 6 months

**Overview:** These studies will be completed in 2003-04.

## KEY RESEARCH ACCOMPLISHMENTS-2002-03

- A reversed phase HPLC assay and stability studies for NITC were published.

- The pharmacokinetics and metabolism of 1-NITC were characterized in rats. This study represents the first determination of the pharmacokinetics of α-naphthylisothiocyanate. This manuscript is in preparation.

- A LC/MS/MS assay for PEITC was developed. This novel assay represents the first analytical method with the sensitivity and specificity to determine plasma and urine concentrations of PEITC. This manuscript has been submitted for publication.

- The pharmacokinetics of PEITC have been determined in rats. We have shown for the first time that the clearance of PEITC is dose dependent, and that following oral administration of PEITC, the bioavailability is high.

- We have demonstrated that although PEITC can inhibit the efflux of daunomycin and vinblastine by P-glycoprotein, its own cellular accumulation is not altered in MCF-7adr or MDA-LCC6/mdr1 cells (which overexpress P-glycoprotein) by the P-glycoprotein inhibitor verapamil. This suggests that PEITC may not represent a substrate for P-glycoprotein. Additionally, the isocyanate and amine metabolites of PEITC, BITC and NITC do not affect P-glycoprotein-mediated efflux.

- We have published a review paper on gender differences in membrane transport to evaluate potential differences in transport by P-glycoprotein and other transport proteins in women and men. This represents an important consideration in the interindividual differences in pharmacokinetics and efficacy of drugs.

- We have written and submitted a review paper on efflux transporters in drug elimination, which includes a review of P-glycoprotein, MRP transporters and breast cancer resistance protein, all potential targets of isothiocyanates.

## REPORTABLE OUTCOMES

Manuscripts:

Published (in Appendix A)

1. Tseng E, Kamath AV, Zhang S and Morris ME. Effect of organic isothiocyanates on the P-glycoprotein and MRP1-mediated transport of daunomycin and vinblastine, Pharm Res 19:1509-1515, 2002.

2. Morris ME, Lee H-J, Predko LM. Gender differences in the membrane transport of endogenous and exogenous compounds, Pharmacol Rev 55:229-240, 2003.

3. Hu K and Morris ME: Determination of 1-naphthylisothiocyanate (1-NITC) and metabolites 1-naphthylamine (1-NA) and 1-naphthylisocyanate (1-NIC) in rat plasma and urine

by high-performance liquid chromatography, J Chromatography B 788:17-28,2003.

Submitted (in Appendix B)
4. Zhang S and Morris ME: Efflux Transporters in Drug Excretion. Invited Review for Frontiers of Biotechnology and Pharmaceuticals, submitted May, 2003.

5. Ji Y and Morris ME: Determination of Phenethyl Isothiocyanate in Human Plasma and Urine by Ammonia Derivatization and Liquid Chromatography-Tandem Mass Spectroscopy. Submitted 5/26/03.

Abstracts (in Appendix C):

1. Determination of α-naphthylisothiocyanate and its metabolite α-naphthylamine in rat plasma and urine by high-performance liquid chromatographic assay. K. Hu and M. E. Morris, American Association of Pharmaceutical Scientists Annual Meeting, Toronto, Onatario, November 8-12, 2002.
2. Effect of organic isothiocyanates on the p-glycoprotein and MRP1-mediated transport of daunomycin and vinblastine, E. Tseng, A. Kamath, and M.E. Morris. Era of Hope 2002 Meeting.
3. Effects of benzyl-, phenethyl- and alpha-naphthyl isothiocyanates on P-glycoprotein- and MRP1-mediated transport of daunomycin, Hu K and Morris ME. Submitted to the American Association of Pharmaceutical Scientists Annual Meeting, Salt Lake City UT, October 26-30, 2003.
4. Pharmacokinetics of α-naphthylisothiocyanate in rats, Hu K and Morris ME. Submitted to the American Association of Pharmaceutical Scientists Annual Meeting, Salt Lake City UT, October 26-30, 2003.

Graduate Students Participating in this Research as a part of their educational program during 2002-03:

Yushin Kuo, M.S. Candidate (degree expected, Sept, 2003)
Yan Ji, Ph.D. candidate (degree expected, 2005)
Shuzhong Zhang, Ph.D. candidate (degree expected, 2004)

Undergraduate Students Participating in this Research:

Heather Rochette (Biomedical Sciences Major)-Research rotation

Postdoctoral fellow Participating in this Research

Ke Hu

## CONCLUSIONS

1. One strategy to enhance the effectiveness of cancer chemotherapy is to reverse the MDR phenomena. Our results indicate that certain dietary ITCs inhibit the P-gp-mediated efflux of DNM and VBL in MDR breast cancer cells. These compounds have direct inhibitory effects, although PEITC does not represent a P-glycoprotein

substrate. Our recent studies has shown that the isocyanate and amine metabolites of PEITC, BITC and NITC do not inhibit P-glycoprotein-mediated efflux, and that PEITC does not appear to be a substrate for P-glycoprotein.

2. HPLC assays were developed to determine PEITC, BITC and NITC concentrations in biological fluids. A novel LC/MS/MS method for PEITC was developed with the specificity and sensitivity to measure PEITC in biological fluids.

3. The pharmacokinetics and metabolism of NITC were determined in rats. 1-NITC exhibited nonlinear Michaelis Menten disposition: maximium velocity ($V_{max}$), 2.13 ± 0.20 mg/h/kg; Michaelis Menten constant ($K_m$), 0.51 ± 0.13 mg/L.

4. The pharmacokinetics and bioavailability of PEITC were determined in rats. The clearance of PEITC was dose dependent, but PEITC exhibited excellent bioavailability following oral administration.

## REFERENCES

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# Appendices

## Appendix A –Publications

1. Tseng E, Kamath AV, Zhang S and Morris ME. Effect of organic isothiocyanates on the P-glycoprotein and MRP1-mediated transport of daunomycin and vinblastine, Pharm Res 19:1509-1515, 2002.
2. Morris ME, Lee H-J, Predko LM. Gender differences in the membrane transport of endogenous and exogenous compounds, Pharmacol Rev 55:229-240, 2003.
3. Hu K and Morris ME: Determination of 1-naphthylisothiocyanate (1-NIC) and metabolites 1-naphthylamine (1-NA) and 1-naphthylisocyanate (1-NIC) in rat plasma and urine by high-performance liquid chromatography, J Chromatography B 788:17-28,2003.

## Appendix B –Submitted Manuscripts

1. Zhang S and Morris ME: Efflux Transporters in Drug Excretion. Invited Review for Frontiers of Biotechnology and Pharmaceuticals, submitted May, 2003.
2. Ji Y and Morris ME: Determination of Phenethyl Isothiocyanate in Human Plasma and Urine by Ammonia Derivatization and Liquid Chromatography-Tandem Mass Spectroscopy. Submitted 5/26/03.

## Appendix C -Abstracts-published and submitted

1. Determination of α-naphthylisothiocyanate and its metabolite α-naphthylamine in rat plasma and urine by high-performance liquid chromatographic assay. K. Hu and M. E. Morris, American Association of Pharmaceutical Scientists Annual Meeting, Toronto, Onatario, November 8-12, 2002.
2. Effect of organic isothiocyanates on the p-glycoprotein and MRP1-mediated transport of daunomycin and vinblastine, E. Tseng, A. Kamath, and M.E. Morris. Era of Hope 2002 Meeting.
3. Effects of benzyl-, phenethyl- and alpha-naphthy isothiocyanates on P-glycoprotein- and MRP1-mediated transport of daunomycin, Hu K and Morris ME. Submitted to the American Association of Pharmaceutical Scientists Annual Meeting, Salt Lake City UT, October 26-30, 2003.
4. Pharmacokinetics of α-naphthylisothiocyanate in rats, Hu K and Morris ME. Submitted to the American Association of Pharmaceutical Scientists Annual Meeting, Salt Lake City UT, October 26-30, 2003.

*Pharmaceutical Research, Vol. 19, No. 10, October 2002 (© 2002)* *Research Paper*

# Effect of Organic Isothiocyanates on the P-Glycoprotein- and MRP1-Mediated Transport of Daunomycin and Vinblastine

**Elaine Tseng,[1,2] Amrita Kamath,[1,3] and Marilyn E. Morris[1,4]**

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***Purpose.*** Organic isothiocyanates (ITCs), or mustard oils, are nonnutrient components present in the diet, especially in cruciferous vegetables. The purpose of this investigation was to examine the effect of ITCs on P-glycoprotein (P-gp)- and multidrug resistance-associated Protein (MRP1)-mediated transport in multidrug resistant (MDR) human cancer cell lines.

□ □□□□□The direct effect of ITCs on the 2-h cellular accumulation of daunomycin (DNM) and vinblastine (VBL), substrates for both P-gp and MRP1, were measured in sensitive and resistant MCF-7 cells and in PANC-1 cells. Resistant MCF-7 cells (MCF-7/ADR) overexpress P-gp whereas PANC-1 cells overexpress MRP1. The following compounds were evaluated: allyl-, benzyl-(BITC), hexyl-, phenethyl-(PEITC), phenyl-, 1-naphthyl-(NITC), phenylhexyl-, phenylpropyl-, and phenylbutyl-ITC, sulforaphane, erucin, and erysolin.

□ □□□□□NITC significantly increased the accumulation of DNM and VBL in both resistant cell lines, but had no effect on DNM accumulation in sensitive MCF-7 cells. VBL accumulation in resistant MCF-7 cells was increased 40-fold by NITC whereas that in PANC-1 cells was increased 5.5-fold. Significant effects on the accumulation of DNM and VBL in resistant MCF-7 cells were also observed with benzyl-isothiocyanate whereas PEITC, erysolin, phenylhexyl-ITC, and phenylbutyl-ITC increased the accumulation of DNM and/or VBL in PANC-1 cells. Overall, the inhibitory activities of these compounds in MCF-7 cells and PANC-1 cells were significantly correlated ($r^2 = 0.77$ and 0.86 for DNM and VBL, respectively). Significant effects on accumulation were generally observed with the ITCs at 50 μM concentrations, but not at 10 μM concentrations.

□ □□□□□□□□□ One strategy to enhance the effectiveness of cancer chemotherapy is to reverse the MDR phenomena. Our results indicate that certain dietary ITCs inhibit the P-gp- and the MRP1-mediated efflux of DNM and VBL in MDR cancer cells and suggest the potential for diet-drug interactions.

**KEY WORDS:** multidrug resistance; phenethylisothiocyanate; benzylisothiocyanate; naphthylisothiocyanate; cancer chemotherapy.

## INTRODUCTION

What may be considered a major setback from successful cancer chemotherapy is the phenomenon of simultaneous resistance to many structurally unrelated cytotoxic agents known as multidrug resistance (MDR; 1). One well-characterized mechanism is the overexpression of efflux proteins at the surface of the cell membrane, including p-glycoprotein (P-gp) and multidrug resistance–associated protein (MRP1). Overexpression of P-gp and/or MRP1 results in the increased efflux and therefore decreased intracellular concentrations of many natural product chemotherapeutic agents. These efflux pumps may be present at the time of diagnosis and/or may be overexpressed after drug exposure.

P-glycoprotein-mediated efflux is one mechanism of MDR that has been extensively studied. The 170kD P-gp encoded by the MDR1 gene belongs to the ATP-binding cassette (ABC) superfamily of proteins (ABCB1) and functions as an ATP-dependent efflux pump responsible for the transfer of a wide variety of xenobiotics and carcinogens from cells (2). The diverse classes of antitumor drugs that are P-gp substrates include anthracyclines, vinca alkaloids, epipodophyllotoxins, and taxanes. Besides being overexpressed in various tumor cells (3), P-gp is expressed endogenously in adrenal tissues, kidney, lung, liver, and colon (4). The differential expression of P-gp in normal tissues and its conservation among species suggest that the protein many have distinct physiologic roles associated with specialized cell functions. The tissue distribution of P-gp, mainly in the epithelia of excretory organs, and the ability to transport a wide range of lipophilic substrates, are compatible with the hypothesis that P-gp serves a detoxification function in the body. In cancer cells, the overexpression of P-gp decreases the intracellular concentrations of chemotherapeutic drugs and has been positively correlated with poor prognosis in cancers (2).

Overexpression of the 190-kd multidrug resistance-associated protein (MRP1) encoded by the MRP1 gene in cancer cells also results in MDR. Although first characterized in small cell lung cancer cells (5), MRP1 is present in almost all cells of the human body, as well as overexpressed in non-P-gp MDR cell lines of the lung, colon, gastric, ovary, and breast (6). MRP1 also belongs to the family of ABC membrane transporters (ABCC1), and in a similar manner as P-gp, mediates resistance to a range of structurally and functionally unrelated agents (7). However, whereas P-gp and MRP1 both transport a number of natural product chemotherapeutic agents, substrate preferences do exist. The preferred substrates for MRP1 are usually organic anions, in particular, drugs conjugated with glutathione (GSH), glucuronate, or sulfate. In fact, MRP acts as a GS-X pump, transporting drugs conjugated to GSH out of the cell (7).

The identification and characterization of these two efflux pumps in MDR has stimulated extensive research into the search for clinically useful inhibitors. Although many inhibitors including calcium channel blockers (e.g., verapamil, nifedipine), hypotensive drugs (reserpine), antibiotics (cephalosporins, gramicidin, puromycin), immunosuppressors (cyclosporinA and its derivatives), and many other lipophilic compounds have been identified and investigated, clinical trials have been largely unsuccessful as a result of dose-related toxicities that occur at the doses necessary to achieve MDR reversal (8).

The main objective of the present study was to examine the effects of dietary organic isothiocyanates (ITCs) on P-gp-

[1] Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, The State University of New York at Buffalo, Amherst, New York 14260.

[2] Current address: Groton Laboratories, Pfizer Inc., Eastern Point Road 8220-2356, Groton, Connecticut 06340.

[3] Current address: Bristol Myers Squibb, P.O. Box 4000, Princeton, New Jersey 08543.

[4] To whom correspondence should be addressed. (e-mail: memorris@acsu.buffalo.edu)



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and MRP1-mediated transport of chemotherapeutic agents in human cancer cell lines. Organic isothiocyanates (and glucosinolates, the biosynthetic precursors of ITCs in plants), also known as mustard oils, are widely distributed in edible plants, including cruciferous vegetables, with human consumption estimated at milligram quantities daily. Glucosinolate levels have been estimated to be as high as 180 mg/g of some vegetables (9). In the present investigation we examined the effects of a range of natural and synthetic ITCs on the cellular accumulation of the P-gp and MRP1 substrates, daunomycin (DNM) and vinblastine (VBL) after 2-h exposure times. Studies were performed in sensitive and resistant human breast cancer cells (MCF-7) and human pancreatic cancer cells (PANC-1). Resistant MCF-7 cells (MCF-7/ADR) overexpress P-gp whereas PANC-1 cells overexpress MRP1.

## MATERIALS AND METHODS

Erysolin, phenyl ITC, β-phenylethyl ITC, α-naphthyl ITC, and verapamil were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Benzyl ITC, n-hexyl ITC, and allyl ITC were obtained from Aldrich (St. Louis, MO, USA). Sulforaphane and erucin were purchased from ICN (Aurora, OH, USA), and phenylpropyl ITC and phenybutyl ITC were purchased from LKT Laboratories (St. Paul, MN, USA). Phenylhexyl ITC was a gift from National Cancer Institute-Chemopreventive Division (Bethesda, MD, USA). Radiolabeled [$^3$H]-daunomycin (14.4 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, USA), and [$^3$H]-vinblastine sulfate (7.3 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). Cell culture reagents were supplied by GIBCO BRL (Buffalo, NY, USA), and cell culture flasks and dishes were purchased from Falcon (Becton Dickinson, Franklin Lakes, NJ, USA). Biodegradable liquid scintillation cocktail was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Commassie blue dye reagent was obtained from Bio-Rad laboratory (Hercules, CA, USA). The MCF-7 and MCF-7/ADR cell lines were gifts from Dr. Ralph Bernacki (Roswell Park Cancer Institute). The PANC-1 cell line was obtained from American Type Culture Collection (Manassas, VA, UA). The monoclonal antibodies C219 and MRPr1 were obtained from Kamiya Biomedical Co. (Seattle, WA, USA).

### Western Analysis of P-gp and MRP1

P-gp and MRP1 expression in the cells was determined by Western analysis using the antibodies C219 and MRPr1 as described previously (10). The protein molecular weight markers (Rainbow Markers, Amersham) used were myosin (200 kd), phosphorylase b (97.4 kd), and ovalbumin (46 kd). Membrane preparations from MCF-7 and PANC-1 cells were isolated using the method of Wils *et al.* (11). Protein concentrations were measured by the Bradford method (12) using a commercially available assay kit (Bio-Rad Labs) with γ-globulin as the standard. Proteins were electrophoresed on 7.5% SDS-polyacrylamide gels and electroblotted on nitrocellulose filter. The filter was blocked overnight at 4°C in Tris-buffered saline containing 0.2% (v/v) Tween 20 and 1% (w/v) bovine serum albumin, incubated with C219 (1 μg/mL) or MRPr1 (1:30) antibodies in blocking buffer for 2 h at room temperature. The filters were then washed in washing buffer (20 mM Tris base, 137 mM NaCl, 1% Tween 20, pH 7.6) and incubated with 1:1500 (v/v) anti-mouse IgG HRP secondary antibody (Amersham; for C219) or 1:1000 anti-rat IgG HRP secondary antibody (Zymed, San Francisco, CA, USA; for MRPr1), in blocking buffer for 2 h. After washing, the protein was detected using the ECL detection reagent (Amersham). Kodak 1D image analysis software was used to analyze the Western blot results.

### Cell Culture

MCF-7 and MCF-7/ADR, used between passages 16–24, were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (10 units/mL), and streptomycin (10 μg/mL). Cells were incubated at 37°C supplemented with 5% $CO_2$/95% air. Cells were subcultured two to three times a week using 0.05% trypsin-0.53 mM EDTA. Cells were grown in 75-cm$^2$ plastic culture flasks that were seeded in 35-mm$^2$ plastic culture dishes for accumulation studies. Experiments were performed 2 to 3 days after seeding.

PANC-1 cells used between passages 60–75 were grown in Dulbecco's modified Eagle's medium supplemented with L-glutamine, sodium pyruvate, pyridoxine HCl, and 10% fetal bovine serum, which was maintained in an atmosphere of 10% $CO_2$/90% air at 37°C. Cells were subcultured every 2 to 3 days with 0.25% trypsin-2.6 mM EDTA. For experiments, cells were seeded on 35-mm$^2$ dishes at a density of $10^6$ cells per dish and used 2 days later.

### Accumulation Studies

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 $MgCl_2 \cdot 6H_20$, 10 mM HEPES, pH 7.4). One milliliter of incubation buffer containing 0.05 μM of [$^3$H]-DNM or 0.05 μM [$^3$H]-VBL and 100 μM of ITC was added to the dish and incubated for 2 h. Verapamil, a P-gp and MRP1 inhibitor, was used as a positive control in all studies. Concentration-dependent studies were performed with some of the ITCs using concentrations varying from 100 to 0.1 μM. The uptake 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 milliliter of 0.5% Triton-X-100 or 0.3 N NaOH-1%SDS was added to each dish, and aliquots were obtained after an hour. A liquid scintillation counter (1900 CA, Tri-Carb liquid scintillation analyzer, Packard Instruments Co.) was used to determine the radioactivity. The protein concentration was determined by the Bradford method (12) using a commercially available assay kit (Bio-Rad Labs) with γ-globulin as the standard.

### Data Analysis

Statistical significance was determined by a one-way ANOVA followed by Dunnett's post hoc test. Differences were considered to be significant when $p < 0.05$.

## RESULTS

### MCF-7 Cells

*Western Analysis*

Western blot analyses were preformed to evaluate P-gp and MRP1 expression in MCF-7/WT, MCF-7/ADR, and

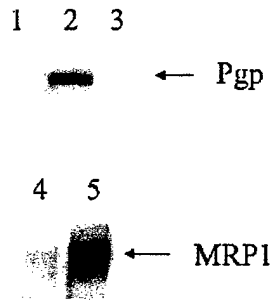
PANC-1 cells. There were undetectable amounts of P-gp in the MCF-7/WT and PANC-1 cell lines but high expression in the MCF-7/ADR cell line. PANC-1 cells showed high expression of MRP1. MCF-7/ADR cells also exhibited low expression of MRP1 (Fig. 1). The results found in this experiment confirmed those in the literature (13,14).

*Time Course Study*

The time course of uptake of 0.05 μM $^3$H-DNM in the presence and absence of 100 μM verapamil, a typical inhibitor, was examined in sensitive (MCF-7/WT) and resistant (MCF-7/ADR) cells for up to 2 h (Fig. 2). For MCF-7/ADR cells, the accumulation of DNM was significantly greater in the presence of verapamil when compared with that in the absence of verapamil. In the sensitive cell line, which lacks P-gp, accumulation of DNM in the presence or absence of verapamil was unchanged; this demonstrates that verapamil influences the efflux of DNM through the inhibition of P-gp and not through other mechanisms in this cell line. Equilibrium conditions were achieved by 2 h in both the sensitive and resistant MCF-7 cells.

*DNM Accumulation*

The effect of various organic ITCs on DNM accumulation was examined in MCF-7/WT cells (Fig. 3). Verapamil did not significantly increase DNM accumulation in the sensitive cells. Only phenylpropyl ITC and phenylhexyl ITC produced significant increases in DNM accumulation in these cells. In MCF-7/ADR cells, verapamil was able to significantly increase DNM accumulation by 2.5-fold compared with the control. Few ITCs were found to inhibit the efflux of DNM, with the most active compound being 1-naphthylisothiocyanate (NITC), which increased DNM accumulation by 4-fold; benzylisothiocyanante (BITC) produced an effect that was similar in magnitude to that of verapamil 100 μM. All other compounds did not significantly alter DNM accumulation. Concentration-dependent studies demonstrated significant activity for NITC at concentrations of 50 μM but not at 10 μM (results not shown).




1. MCF-7/sensitive 2. MCF-7/ADR
3. PANC-1 4. MCF-7/ADR
5. PANC-1

**Fig. 1.** Western blots of P-gp and MRP1 in MCF-7, MCF-7/ADR, and PANC-1 cells, using the antibodies C219 and MRPr1, respectively (as described in the Materials and Methods section).

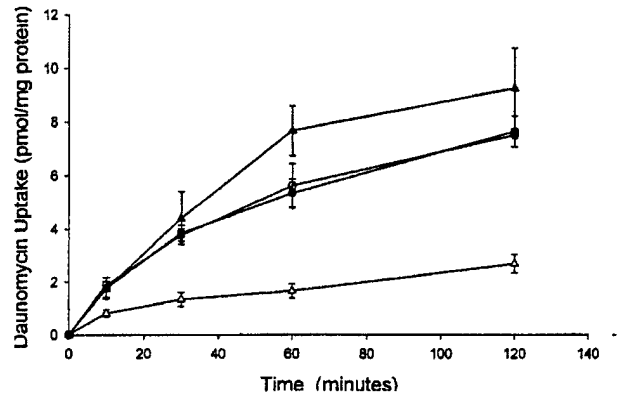



**Fig. 2.** Time course of daunomycin uptake in MCF-7 sensitive and resistant cells. DNM (0.05 μM) uptake was measured in the presence and absence of verapamil. (▲) MCF-7/ADR + verapamil (100 μM), (△) MCF-7/ADR control, (●) MCF-7/WT + verapamil (100 μM), (○) MCF-7/WT control. Data are mean ± SD of data from one representative study. The study was repeated with similar results.

*VBL Accumulation*

The uptake of VBL was examined in the presence and absence of ITCs. In MCF-7/ADR cells, verapamil signifi-

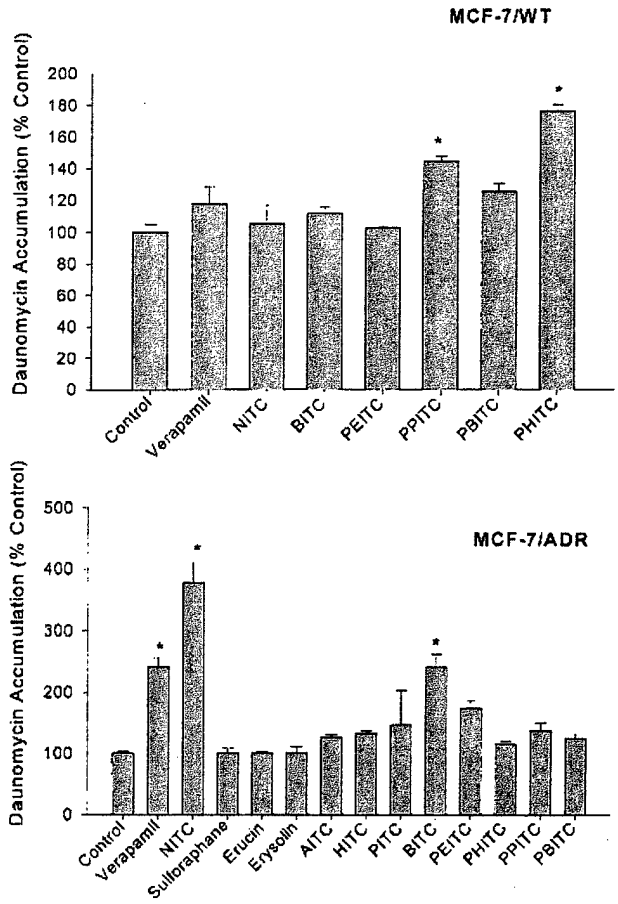



**Fig. 3.** Effect of organic isothiocyanates (ITCs) on daunomycin accumulation in MCF-7 cells. The 2-h accumulation of 0.05 μM daunomycin was measured in the presence of various ITCs (100 μM). Control represents the uptake in the absence of ITCs. Each bar represents mean ± SE, n = 9–12, *p < 0.001.

cantly increased the accumulation of VBL by 33-fold, phenylhexyl ITC by 10-fold, and NITC by 40-fold (Fig. 4). The greatest effects of the ITCs on accumulation were seen for VBL in MCF-7/ADR cells.

## PANC-1 Cells

### *DNM Accumulation*

In PANC-1 cells, phenethylisothiocyanate (PEITC), erysolin, NITC, and verapamil were able to significantly increase DNM accumulation (Fig. 5). A number of other ITCs, including BITC, allyl ITC, and hexyl ITC, demonstrated a trend towards increased accumulation of DNM ($p < 0.1$). Concentration-dependent studies demonstrated significant activity for NITC and PEITC at 50 μM concentrations but not at 10 μM concentrations (results not shown).

### *VBL Accumulation*

Verapamil was able to significantly increase VBL accumulation by 4-fold. The ITCs that demonstrated significant effects were: NITC (5.5-fold), PEITC (2-fold), phenylhexyl ITC (3-fold), and phenylbutyl ITC (2.5-fold). All other compounds did not have significant effects, although a number showed a trend towards significance, including BITC, allyl ITC, and hexyl ITC (Fig. 6). The correlation between ITC inhibition (percent control values) for DNM and VBL in PANC-1 cells had an $r^2$ value of 0.37 ($p < 0.05$; not shown.)

We also examined the correlation between ITC inhibition in MCF-7 cells and PANC-1 cells. The ITC-mediated changes in cellular accumulation for both DNM and VBL in MCF-7/ADR and PANC-1 cells were highly correlated with $r^2$ values of 0.77 for DNM ($p < 0.05$; Fig. 7A) and 0.86 for VBL ($p < 0.005$; Fig. 7B).

## DISCUSSION

Drug resistance represents a major cause for therapeutic failure and death in cancer treatment. An important mechanism of this resistance is the enhanced cellular efflux of a wide variety of structurally distinct classes of chemotherapeutic agents because of the overexpression of P-gp and/or MRP1. Studies of biopsy samples from patients have revealed elevated levels of *P*-gp in tumors of every histologic type, with a strong association in leukemias, lymphomas, and some childhood solid tumors between the detection of tumor P-gp and poor response to therapy (15). MRP1 has been identified in a number of different cancers (16): in neuroblastoma, MRP1 levels are elevated and are significantly correlated with N-myc, a negative prognostic factor for response to chemotherapy in neuroblastoma patients. Buser *et al.* (17) reported a high prevalence of P-gp in breast cancer tumor tissue: 83% in early breast cancer and 100% in primarily metastatic breast cancer. One strategy for reversing MDR in cancer has been

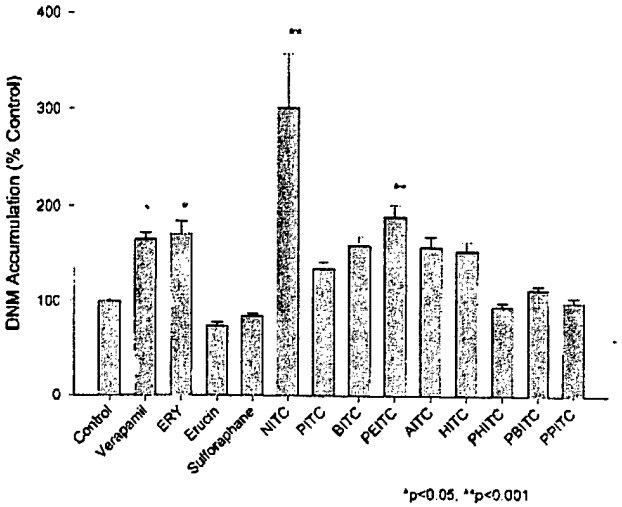



**Fig. 5.** Effect of organic isothiocyanates (ITCs) on daunomycin accumulation in PANC-1 cells. The 2-h accumulation of 0.05 μM daunomycin was measured in the presence of various ITCs (100 μM). Control represents the uptake in the absence of ITCs. Each bar represents mean ± SE, n = 9–12, *$p < 0.05$ **$p < 0.001$.

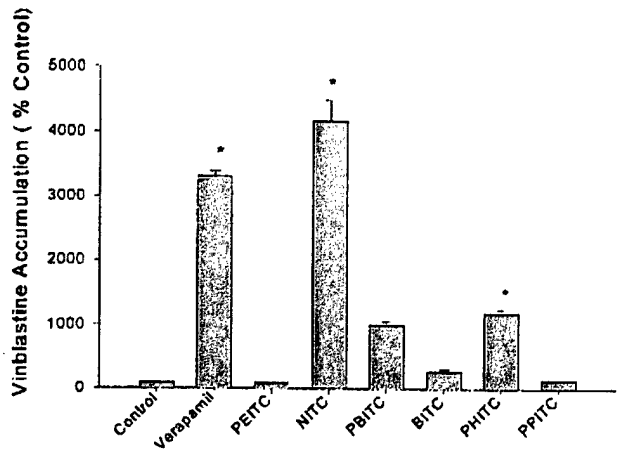



**Fig. 4.** Effect of organic isothiocyanates (ITCs) on vinblastine accumulation in MCF-7/ADR cells. The 2-h accumulation of 0.05 μM vinblastine was measured in the presence of various ITCs (100 μM). Control represents the uptake in the absence of ITCs. Each bar represents mean ± SE, n = 9–12, *$p < 0.001$.

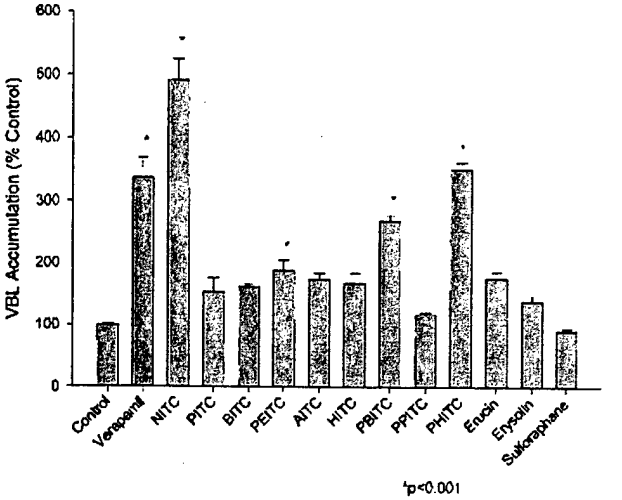



**Fig. 6.** Effect of organic isothiocyanates (ITCs) on vinblastine accumulation in PANC-1 cells. The 2-h accumulation of 0.05 μM vinblastine was measured in the presence of various ITCs (100 μM). Control represents the uptake in the absence of ITCs. Each bar represents mean ± SE, n = 9–12, * $p < 0.001$.

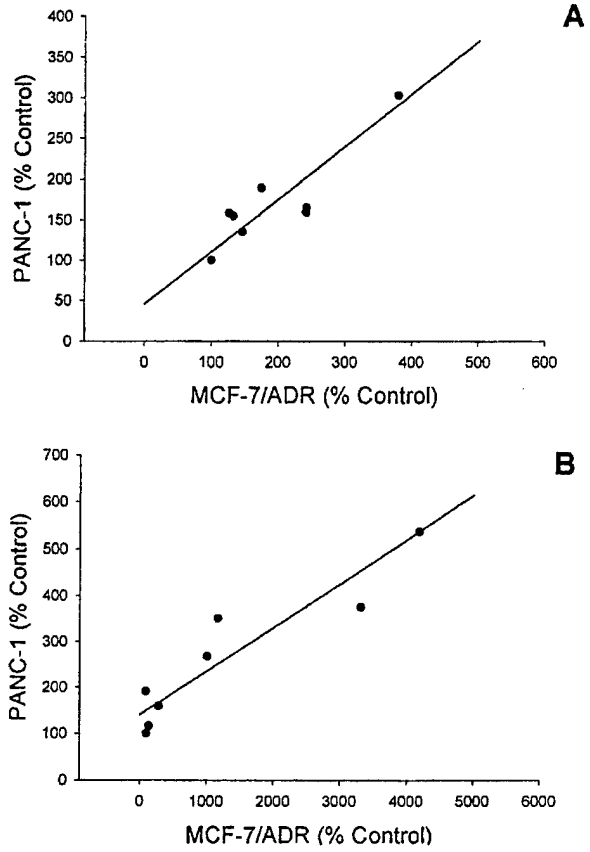



**Fig. 7.** Correlation between ITC inhibition in MCF-7/ADR cells and PANC-1 cells. (A) The relationship between ITC inhibition of daunomycin in MCF-7/ADR cells with that in PANC-1 cells. $r^2 = 0.77$, $p < 0.05$. (B) The relationship between ITC inhibition of vinblastine in MCF-7/ADR cells with that in PANC-1 cells. $r^2 = 0.86$, $p < 0.005$.

the concomitant use of chemical agents that are by themselves nontoxic but that increase the accumulation of chemotherapeutic drugs in MDR cells through the inhibition of P-gp- or MRP1-mediated efflux of these agents.

In this study, we investigated a class of dietary compounds, the organic ITCs, as inhibitors of P-gp- and MRP1-mediated drug resistance in cancer cell lines. The organic ITCs are components present in the diet, especially in cruciferous vegetables such as broccoli, watercress, cabbage, and brussel sprouts. Numerous experiments have reported that ITCs can inhibit tumor formation of the skin, lung, colon, and breast in animal models (9,18,19), although the mechanism by which this happens is still not completely understood. ITCs are currently being evaluated in clinical trials for the prevention of lung cancer (19). There is substantial evidence that the inhibition of tumorigenesis is partly the result of the direct inhibition and/or downregulation of the CYP-450s responsible for carcinogen activation (16). In addition, ITCs can induce phase II enzymes responsible for the detoxification of electrophilic intermediates formed during phase I metabolism (19). Other mechanisms are likely involved in the chemopreventive effects of ITCs: recent studies have indicated that sulforaphane induces cell cycle arrest and apoptosis in HT29 human colon cancer cells (20) and PEITC also induces apoptosis in cells (21).

Although the organic isothiocyanates represent a group of lipophilic natural products, they have not previously been investigated as substrates or inhibitors of P-gp or MRP1. We have found that NITC and BITC can increase the accumulation of DNM and VBL in the drug-resistant human breast cancer cell line MCF-7 without affecting accumulation in sensitive MCF-7 cells. Interestingly, two of the ITCs tested, phenylpropyl ITC and phenylhexyl ITC, significantly increased the accumulation of DNM in the MCF-7/WT cells but not in the MCF-7/ADR cells. The mechanism underlying this interaction is unknown. Additionally, a number of organic ITCs, including NITC and PEITC, increased the 2-h accumulation of DNM and VBL in PANC-1 cells, which overexpress MRP1 but not P-gp. At this time, it is not known whether these compounds represent substrates for P-gp or MRP1 or whether they are only inhibitors. Because the effects occur rapidly, this suggests that the inhibition might involve a direct interaction at the binding site or at an allosteric site that affects the binding of DNM or VBL. P-gp has been reported to have more than one substrate-binding site. Shapiro and Ling (22) reported that P-gp contains three distinct sites for drug binding, one which transports rhodamine 123, a second that transports Hoechst 33342, and a third that is specific for prazosin or progesterone (23). The anthracyclines inhibit rhodamine 123 transport and stimulate Hoechst 33342 transport whereas VBL, actinomycin D, and etoposide inhibit transport of both dyes. This suggests that compounds like DNM may represent a substrate for only one site whereas VBL may be a substrate for more than one site.

Substrates for MRP1 are endogenous and exogenous organic anions that are conjugated by glutathione, glucuronide, or sulfate, including leukotriene C4 (cysteinyl leukotrienes), glutathione disulfide (oxidized glutathione), and steroid glucuronides (17β-estradiol 17-β-D-glucuronide; 7). Natural product chemotherapeutic agents that do not form a glutathione conjugate, such as anthracyclines, vinca alkaloids, methotrexate, fluorouracil, and chlorambucil (24) are also substrates for MRP1. These drugs are likely transported by MRP1 in a GSH-dependent manner, which may involve the cotransport of GSH and the chemotherapeutic agent (24). Dietrich *et al.* (25) have demonstrated the MRP2-mediated biliary excretion of NITC, either as a GSH conjugate or in association with GSH, indicating that it is a substrate for MRP2. Our studies have demonstrated that the inhibitory effects of the ITCs on either DNM or VBL accumulation in MCF-7/ADR and PANC-1 cells are highly correlated. This finding was not unexpected because there is overlap in substrate specificity for these transporters, with many of the natural product chemotherapeutic agents being substrates for both transporters.

Our concentration-dependent studies indicate that the ITCs are not potent direct inhibitors of P-gp- or MRP1-mediated efflux. Concentrations of 50 μM of NITC, PEITC, and BITC are effective inhibitors; after a 2-h accumulation study, the compounds were ineffective at a concentration of 10 μM. However, concentration-dependent effects after prolonged exposures have not been examined. After vegetable consumption, concentrations of ITCs in plasma are likely in the nM range (26), although there have been no studies that have determined blood levels of unchanged ITCs. Blood concentrations of ITCs would be expected to vary because of genetic differences in their metabolism by glutathione-S-

transferase M1 and T1 (GSTM1 and GSTT1). Conjugation with glutathione, followed by further conjugation reactions to form the mercapturic conjugate, represents the major route of elimination of PEITC and BITC. GSTM1 and T1 exhibit genetic polymorphisms: 60% of Chinese subjects and 40–50% of people in a variety of ethnic groups are deficient in the GSTM1 gene whereas 10–30% of Europeans are deficient in the GSTT1 polymorphism (27,28). These subjects would be expected to have higher blood concentrations of ITCs than those with the wild-type enzyme. It has been reported that the protective effect of dietary ITC intake for lung cancer risk among current smokers is greatest in individuals null for both GSTM1 and GSTT1 genotypes (27,28). What might be more relevant than plasma ITC concentrations would be intracellular concentrations. Intracellular concentrations of ITCs have been reported to be much higher than extracellular concentrations: for example, cells exposed to 100 μM concentrations of sulforaphane have intracellular concentrations of 6.4 mM, likely as GSH conjugates (29). The relationship between intracellular concentrations and efficacy has not been evaluated for the ITCs.

P-gp and MRP1 also play important roles in the bioavailability, distribution, and elimination of administered drugs (8). In the kidney, P-gp is highly expressed on the brush border of the proximal renal tubule. Speeg *et al.* (30) have demonstrated the inhibition of renal clearance of colchicine by cyclosporin, suggesting that MDR modulators may alter the renal elimination processes of anticancer drugs by blocking P-gp in kidneys. P-gp and some isoforms of MRP are present in the apical membrane of intestinal epithelial cells, where they can limit the absorption of xenobiotics, and in the canalicular membrane of hepatocytes, where they can affect biliary excretion. For example, oral administration of paclitaxel to wild-type and *mdr1a* knockout mice resulted in a 6-fold higher plasma level of paclitaxel in the latter, at least partly as a result of increased bioavailability (31). We found that 100 μM concentrations of NITC, BITC, and PEITC could significantly increase the 2-h accumulation of DNM in the porcine renal cell line LLC-PK1, which expresses low levels of P-gp (Tseng E and Morris ME, unpublished results). Whether this is the result of inhibition of P-gp and/or other transporters is currently unknown. It is likely that exposure to ITCs present in the diet may affect the bioavailability, and possibly disposition, of compounds transported by P-gp and/or MRP1.

The results of this investigation demonstrate for the first time that P-gp and MRP1 activity can be modulated by naturally occurring organic ITCs. Further studies are needed to evaluate the time-dependent nature of this inhibition, and its clinical relevance.

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# Gender Differences in the Membrane Transport of Endogenous and Exogenous Compounds

MARILYN E. MORRIS, HWA-JEONG LEE, AND LISA M. PREDKO

*Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York, Amherst, New York*



*Abstract*——**Gender differences have been well described in pharmacokinetics and contribute to the interindividual variation in drug disposition, therapeutic response, and drug toxicity. Sex-related differences in the membrane transport of endogenous substrates and xenobiotics have been reported in various organs of the body including kidney, liver, intestine, and brain. These gender-related differences in transport systems could also contribute to interindividual variability in pharmacokinetics and pharmacodynamics. This review will focus on current knowledge of gender-associated differences in the transport of endogenous and exogenous compounds in a variety of body organs and will discuss the implications and the clinical significance of these observations.**

## I. Introduction

Gender differences in pharmacokinetics and pharmacodynamics are well documented in animals and humans. Gender is one variable that contributes to differences in pharmacokinetics including absorption, distribution, metabolism, and excretion (Bonate, 1991; Fletcher et al., 1994; Harris et al., 1995). The increased bioavailability of ethanol after oral administration has been reported in women as a result of higher alcohol absorption due to lower gastric alcohol dehydrogenase activity (Fletcher et al., 1994; Harris et al., 1995), and aspirin is absorbed more slowly in men than in women after oral dosing (Harris et al., 1995). The effect of gender on hepatic metabolism has been extensively examined for a number of drugs (Bonate, 1991; Fletcher et al., 1994; Harris et al., 1995). The enzyme, cytochrome P-450 3A4 (CYP 3A4) is involved in the metabolism of over 50% of drugs in clinical use including erythromycin, lidocaine, and midazolam and is also responsible for the hydroxylation of steroid hormones. The activity of CYP 3A4 in women is 1.4 times greater than that in men (Harris et al., 1995; Gleiter and Gundert-Remy, 1996). Conjugation reactions also demonstrate gender-related differences. The glucuronidation of diflunisal and paracetamol is higher in men than in women due to higher glucuronosyl transferase activity in men, with no sex-associated differences in sulfation (Gleiter and Gundert-Remy, 1996). Gender-based differences in protein bind-

Address correspondence to: Dr. Marilyn E. Morris, Department of Pharmaceutical Sciences, 527 Hochstetter Hall, University at Buffalo, Amherst, NY 14260-1200. E-mail: memorris@acsu.buffalo.edu
[1] Current address: College of Pharmacy, Ewha Womans University, 11-1 Daehyun-dong, Seodaemun-gu, Seoul, 120-750, South Korea.
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TABLE 1
*Gender-associated differences in transport systems in humans*

| Compounds | Tissue | Process | Gender Effects | Reference |
|---|---|---|---|---|
| Amantadine | Kidney | Urinary recovery at 48 h | M > F | Wong et al., 1995 |
| | | Renal clearance/weight[a] | M > F | Wong et al., 1995 |
| Uric acid | Kidney | Postsecretory reabsorption | M > F | Anton et al., 1986 |
| Rifamycin SV | Liver | Normal & Gilbert's patients | M < F | Gentile et al., 1985 |
| MDR1 (Pgp) | Liver | Protein expression | M > F | Schuetz et al., 1995 |
| Glucose | Skeletal muscles | Uptake | M < F | Nuutila et al., 1995 |
| Chylomicron Triglyceride | Splanchnic tissues | Uptake | M > F | Nguyen et al., 1996 |
| Palmitate | Splanchnic | Uptake | M > F | Nguyen et al., 1996 |

[a] Renal clearance in males was greater than in females following normalization for body weight, body surface area, or body mass index.

ing have been observed for diazepam, chlordiazepoxide, and imipramine, with nonpregnant women having higher unbound fractions of these drugs compared with men (Harris et al., 1995; Kashuba and Nafziger, 1998). This may be due to the slightly lower concentrations of α-1-acid glycoprotein and lipoprotein reported in women; the plasma concentration of α-1-acid glycoprotein is decreased by estrogen (Beierle et al., 1999). Gender-related differences in drug response have not been extensively studied; however, a gender effect in pharmacodynamics has been well described for psychotropic drugs. The greater improvement and more severe adverse effects in response to antipsychotic drugs such as chlorpromazine and fluspirilene have been reported in women, at least in part, due to differences in estrogen concentrations; estrogen has been shown to act as a dopamine antagonist (Fletcher et al., 1994; Harris et al., 1995). As well, Kaasinen et al. (2001) have reported that women have significantly higher dopamine D2-like receptor binding than men in the frontal cortex, which may contribute to gender-related differences in the incidence, clinical course, or treatment response in neuropsychiatric diseases that are associated with dopaminergic neurotransmission. There is a gender difference in the response to the cholinesterase inhibitors, rivastigmine and physostigmine, in that female rats exhibit a greater inhibition of cholinesterase in the cerebral cortex, hippocampus, and striatum compared with male rats: orchidectomy completely abolished the difference suggesting that a testicular hormone may be suppressing the effect of the cholinesterase inhibitor by affecting its brain uptake or its interaction with cholinesterase (Wang et al., 2000). Women on hemodialysis exhibit lower responses to recombinant erythropoietin than male patients (Ifudu et al., 2001). It is not known if these differences are due to inherent biological differences in response to erythropoietin or due to other factors including differences in endogenous erythropoietin levels (Ifudu et al., 2001). More adverse effects for antihypertensive drugs are reported in women than in men (Harris et al., 1995). The gender-related differences in pharmacokinetics and pharmacodynamics may explain, at least in part, the interindividual variations observed in drug disposition, therapeutic response, and drug toxicity and are of particular concern for those drugs with relatively narrow therapeutic ranges (Harris et al., 1995; Gleiter and Gundert-Remy, 1996).

Facilitated transport systems in the intestine, liver, and kidney have been known to play important roles in the absorption and elimination of a variety of clinically significant drugs (Zhang et al., 1998). Drugs must traverse across biological membranes via simple diffusion or physiological transporters to produce therapeutic efficacy (Levy, 1998). Gender-associated differences in transport processes for endogenous and exogenous substrates have been reported in various organs of the body, including kidney, liver, intestine, and brain, for rats, mice, and humans (Kleinman et al., 1966; Orzes et al., 1985; Anton et al., 1986; Morissette et al., 1990; Uhland-Smith and DeLuca, 1993; Sibug et al., 1996). Table 1 summarizes the gender-associated differences in transport activities in humans, evaluated predominantly in clearance studies, whereas Table 2 summarizes the literature information regarding gender differences in transporter mRNA and/or protein expression in tissues. This review will focus on recent knowledge of gender-associated differences in the transport of endogenous compounds and xenobiotics in a variety of body organs and will discuss the implications and the clinical significance of these findings.

## II. Membrane Transport in Tissues

### *A. Kidney*

There are gender differences in renal handling of both organic and inorganic anions and cations.

*1. Anions.* The renal clearance of *p*-aminohippurate (PAH[1]) is decreased in female rats due to decreases in both the filtered and secreted amounts. In females, the maximal uptake ($V_{max}$) into kidney basolateral membrane vesicles is decreased by 52 ± 9% ($p < 0.05$), and

[1]Abbreviations: PAH, *p*-aminohippurate; Oatp, organic anion transporter polypeptide; TEA, tetraethylammonium; rOCT, rat organic cation transport protein; hOCT, human organic cation transport protein; BBM, brush-border membrane; BLM, basolateral membrane; BSP, sulfobromophthalein; TBS, tetrabromosulfonephthalein; BSP-GSH, glutathione conjugate of sulfobromophthalein; Ntcp, sodium-dependent taurocholate transporter; cLPM, canalicular liver plasma membrane; MRP, multidrug resistance-associated protein; ; Pgp, P-glycoprotein; DA, dopamine; BBB, blood-brain barrier; 5-HT, 5-hydroxytryptamine; FATP-1, fatty acid transport protein-1.

TABLE 2
*Gender differences in transporter expression*

| Transport Protein | Typical Substrates | Gender Difference (Species/Tissue) | Sex-Hormone Treatment | | Reference |
|---|---|---|---|---|---|
| | | | Testosterone | Estradiol | |
| Oatp mRNA/protein | Bromosulfophthalein, taurocholate, ouabain, cortisol, dexamethasone, ajmalinium | Male > female (rat/kidney) male = female (rat/liver) | Strong increase | Decrease | Lu et al., 1996; Simon et al., 1999 |
| rOAT1 protein | PAH, $PGE_2$, urate, salicylate, methotrexate, cAMP, indomethacin, folate | Male > female (rat/kidney) | N.D. | N.D. | Cerrutti et al., 2002 |
| rOCT1 mRNA | Choline, dopamine, epinephrine, serotonin, norepinephrine, MPP, NMN, tyramine | None (rat/kidney) | No effect | No effect | Urakami et al., 1999, 2000 |
| rOCT2 mRNA/protein | Amantadine, TEA, choline, dopamine | Male > female (rat/kidney) | Increase | Moderate decrease | Urakami et al., 1999, 2000 |
| rOCT3 mRNA | Dopamine, guanidine, MPP, TEA | None (rat/kidney) | N.D. | N.D. | Urakami et al., 1999 |
| mdr1a mRNA | Hydrophobic (cationic) compounds, anticancer agents, digoxin, immunosuppressants, steroids | Female > male (rat/liver) | N.D. | N.D. | Piquette-Miller et al., 1998 |
| mdr1b mRNA | Hydrophobic (cationic) compounds, anticancer agents, digoxin, immunosuppressants, steroids | Male > female (rat/liver) female > male (mouse/kidney) | N.D. | N.D. | Schinkel et al., 1994; Piquette-Miller et al., 1998 |
| Mdr2 mRNA | Phospholipids, cholesterol | Female > male (rat/liver) | N.D. | N.D. | Furuya et al., 1994; Salphati and Benet, 1998 |
| MDR total protein | Hydrophobic (cationic) compounds, anticancer agents, digoxin, immunosuppressants, steroids | Female > male (rat/liver) Male > female (human/liver) | N.D. | N.D. | Schuetz et al., 1995; Piquette-Miller et al., 1998 |
| Ntcp mRNA/protein | Bile acids | Male > female (rat/liver) | Decrease | N.D. | Simon et al., 1996, 1999 |
| FATP-1 mRNA | Long chain fatty acids | Female > male (human/skeletal muscle) | N.D. | N.D. | Binnert et al., 2000 |

N.D., not determined; $PGE_2$, prostaglandin $E_2$; MPP, *N*-methyl-4-phenylpyridinium; NMN, *N*-methylnicotinamide.

the Michaelis-Menten constant ($K_m$) for uptake into kidney brush-border membrane vesicles is increased by 163 ± 8% ($p < 0.05$), compared with male rats (Cerrutti et al., 2001). Similar results have been noted in older studies using kidney slices. The transport rate of PAH by kidney slices isolated from male rats is higher than that in female rats with higher $V_{max}$ values compared with female rats (Kleinman et al., 1966; Bowman and Hook, 1972) (Table 3). The rate of accumulation of PAH in renal cortical slices of adult male rats is decreased by castration or by blockade of testosterone receptor sites whereas ovariectomy does not increase the transport of PAH in mature female rats. Furthermore, treatment with estradiol in male rats does not reduce renal tubular transport of PAH whereas chronic (repeated) treatment with testosterone stimulates PAH transport in males more than in females. These results indicate the important role of sex hormones in the renal tubular transport of PAH and suggest distinct renal effects of testosterone compared with estradiol (Braunlich et al., 1993). Similar effects have been reported for the renal tubular transport of Diodrast, amino acids, and thiosulfate (as reviewed by Kleinman et al., 1966).

The urinary excretion of zenarestat, an aldose reductase inhibitor, shows remarkable gender differences in rats and mice (Tanaka et al., 1991, 1992), whereas there is no significant difference between male and female dogs and humans (Tanaka et al., 1992) (Table 4). The ratios of the renal clearance of zenarestat to clearance of zenarestat by glomerular filtration are less than one in male rats and substantially greater than one in female rats. After pretreatment of rats and mice with probenecid, an inhibitor of the active secretion of many organic anions, a marked reduction in the urinary excretion of zenarestat is observed in females but not in males. These results suggest that zenarestat is, at least in part, actively secreted in the kidneys of female rats and mice and active renal tubular secretion of this compound is lacking, or negligible, in male rats and mice (Tanaka et

TABLE 3
*Maximal transport rate in the kidney for p-aminohippuric acid in male and female rats*[a]

| Sex | No. of Animals | Kidney Weight/100 g b.wt. | $T_{mPAH}$ |
|---|---|---|---|
| | | | *mg/(min × g kidney)* |
| Female | 8 | 0.70 ± 0.01 | 0.26 ± 0.01* |
| Male | 3 | 0.71 ± 0.02 | 0.41 ± 0.04* |

* $p < 0.0005$; adapted from Kleinman et al., 1966.
[a] Values are the mean ± S.E.

TABLE 4
*Sex difference in the excretion of zenarestat in animals and humans*[a]

| Species | Sex | Urine | Feces |
|---|---|---|---|
| Rat | Male | 1.5 ± 0.4 | 96.2 ± 1.4 |
| | Female | 46.8 ± 6.2 | 51.2 ± 6.4 |
| Mouse | Male | 3.4 ± 0.7 | 94.2 ± 0.8 |
| | Female | 31.3 ± 5.4 | 66.8 ± 5.7 |
| Dog | Male | 11.9 ± 3.4 | 84.4 ± 3.2 |
| | Female | 9.9 ± 3.4 | 88.9 ± 2.6 |
| Human | Male | 17.7 ± 7.3 | |
| | Female | 22.2 ± 8.8 | |

[a] Values are the mean ± S.D. of three animals, 13 male and 12 female humans expressed as percentage of dose. Adapted from Tanaka et al., 1992.

TABLE 5
*Sex differences in urinary excretion of egualen sodium in rats*[a]

| Parameter | Total Radioactivity after Oral Administration | Unchanged Drug after Oral Administration | Total Metabolites after Oral Administration |
|---|---|---|---|
| Male | | | |
| $C_{max}$ | 43.2 μg/ml | 34.2 μg/ml | 11.3 μg/ml[c] |
| AUC | 540 μg · h/ml | 397 μg eq · h/ml | 143 μg · h/ml[d] |
| Urinary excretion | 57.4%[a] | 2.1%[b] | 52.2%[e] |
| Female | | | |
| $C_{max}$ | 38.7 μg/ml | 36.7 μg/ml | 2.6 μg/ml[c] |
| AUC | 353 μg · h/ml | 326 μg · h/ml | 27 μg · h/ml[d] |
| Urinary excretion | 70.4%[a] | 39.5%[b] | 29.9%[e] |

AUC, area under the curve.
[a] Oral dose of 20 mg/kg [$^{14}$C]egualen sodium. All values are means expressed as micrograms of egualen equivalents (adapted from Sato et al., 2000). Values with the same superscript letter are significantly different, $p < 0.01$.

al., 1991, 1992). In addition, the urinary excretion of zenarestat is decreased in female rats with experimentally induced chronic diabetes mellitus due to a decrease in active secretion, whereas there is an increase in the urinary excretion of the drug in male rats with experimentally induced acute or chronic diabetes, most likely due to a reduction in testosterone levels in diabetic states (Tanaka et al., 1993). Similarly, it has also been reported that there are gender differences in the renal excretion of perfluorooctanoic acid (Hanhijarvi et al., 1982), *S*-pentachlorophenyl-*N*-acetyl-L-cysteine (Smith and Francis, 1983), carnitine (Carter and Stratman, 1982), nilvadipine metabolite (M3) (Terashita et al., 1995) and 1-aminocyclohexanecarboxylic acid (Anton et al., 1986). These compounds are rapidly excreted by an active renal secretion in female rats whereas this secretory mechanism appears to be absent or relatively inactive in male rats (Carter and Stratman, 1982; Hanhijarvi et al., 1982; Smith and Francis, 1983; Anton et al., 1986; Terashita et al., 1995).

Egualen sodium, an antiulcer drug, demonstrates a marked sex-related difference in the urinary excretion of unchanged drug and metabolites in rats. The renal clearance of unchanged drug in male rats is 21 times lower than that in female rats, and the urinary excretion of egualen represented 2.1 and 39.5% of the dose in male and female rats, respectively (Sato et al., 2000) (Table 5). Egualen is secreted in the renal tubules by a probenecid-inhibitable process, which can be inhibited by testosterone. Gonadectomized male rats have a similar renal clearance of egualen as female rats, and treatment of gonadectomized rats with testosterone decreased the renal clearance of egualen (Sato et al., 2000) (Table 6).

Sodium/sulfate cotransport in kidney cortex brush-border (BBM) vesicles and sulfate/anion exchange in basolateral (BLM) vesicles, isolated from female and male guinea pig kidneys, have been studied (Lee et al., 1999a). No statistically significant differences in $K_m$ and $V_{max}$ for uptake were found, although uptake values for female animals tended to be greater; the lack of significance may reflect the small number of animals studied ($n = 4$). Sodium/sulfate cotransport is increased in renal epithelial cells in the presence of estrogen (Lee et al., 1999b). Postmenopausal women demonstrate a decreased renal reabsorption of sulfate compared with premenopausal women, although this was not reversed by estrogen supplementation (Benincosa et al., 1995).

A significant gender-related difference occurs in the renal reabsorption of urate in humans, which is of clinical significance. A significant decrease in tubular urate postsecretory reabsorption in the kidneys of adult women leads to a greater urinary excretion and lower serum urate concentrations compared with adult men. Presecretory reabsorption and tubular secretion of urate are similar in women and men. The mechanism underlying this difference is not known but both the renal handling of uric acid and the serum urate levels are not influenced by plasma 17β-estradiol concentrations (Anton et al., 1986).

Renal organic anion transporting polypeptide (oatp) mRNA expression is higher in male rat kidney than in female kidney and has been shown to be under the control of androgen and to a lesser extent estrogen (Lu et al., 1996). It is speculated that the regulation of kidney oatp expression may be necessary for modulating the renal tubular secretion of conjugated estradiol. Five forms of oatp are expressed in rat kidney. Oatp1 has a wide substrate specificity and substrates include conjugated and unconjugated bile acids, steroid hormones, organic anions such as bromosulfophthalein, and bulky organic cations such as *N*-(4,4-azo-*n*-pentyl)-21-deoxyajmalinium. OATs are multispecific organic anion trans-

TABLE 6
*Gender-related differences in renal clearance of egualen sodium in rats*[a]

| | Unchanged Drug |
|---|---|
| | *ml/min/kg* |
| Male | |
| Renal clearance | 0.009 (0.002) |
| Probenecid tx | 0.031 (0.005) |
| Gonadectomy | 0.158 (0.024) |
| Probenecid tx after gonadectomy | 0.055 (0.004) |
| Testosterone tx after gonadectomy | 0.004 (0.002) |
| Female | |
| Renal clearance | 0.193 (0.030)** |
| Probenecid tx | 0.081 (0.009) |
| Gonadectomy | 0.272 (0.055) |
| Testosterone tx after gonadectomy | 0.102 (0.033) |

** $p < 0.01$. tx, treatment.
[a] IV infusion: rate = 4 μg/min. Results expressed as mean (S.E.) (adapted from (Sato et al., 2000).

porters, with all members of the OAT family expressed in the kidney (Sekine et al., 2000). The substrates include endogenous compounds such as prostaglandins, urate and dicarboxylic acids, as well as organic anion drugs including PAH, salicylate, enalapril, and penicillin G (Dresser et al., 2001). Urakami et al. (1999) reported no significant gender-related differences in rat kidney organic anion transporter 1 (rOAT1) mRNA, but Cerrutti et al. (2002) found a significantly lower level of rOAT1 protein expression in rat kidney cortex BLM in females (40% compared with males). The lower expression of rOAT1 in kidney cortex BLM may be responsible, at least in part, for the decreased PAH secretion observed in female rats. Additionally, kidney cortex BBM isolated from female rats exhibit an increased membrane fluidity compared with BBM from male rats (Cerrutti et al., 2002); this may also contribute to the gender differences in membrane transport of substrates.

*2. Cations.* Tetraethylammonium (TEA) accumulation into renal cortical slices from male rats is significantly greater than that from female rats, suggesting a gender difference in the active secretion of hydrophilic organic cations (Bowman and Hook, 1972). TEA uptake into kidney slices from male and female rats is significantly increased with testosterone treatment; estradiol treatment decreased TEA uptake in kidney slices from male rats but not female rats (Urakami et al., 2000) (Fig. 1). The apparent $K_m$ for distal tubular amantadine transport in female rats is significantly higher than that in male rats whereas the value for amantadine transport in isolated proximal tubules is not different in male and female rats. In addition, apparent $V_{max}$ estimates for amantadine uptake in proximal tubules and distal tubules are not significantly different between males and females (Wong et al., 1993). However, a small number of rats were used in this study and significant differences in transport may have been missed.

Rat organic cation transport proteins (rOCT) are present in the kidney and are responsible for the transport of a number of organic cations, including TEA, $N^1$-methylnicotinamide, choline, and dopamine. Expression levels of rOCT2 mRNA and protein in the male rat kidney are much higher than in females; there was no difference in rOCT1 or rOCT3 expression (Urakami et al., 1999, 2000). Treatment of male and female rats with testosterone significantly increased the expression of rOCT2 mRNA and protein in kidney and increased the TEA accumulation in kidney slices. Estradiol treatment produced a moderate decrease in kidney rOCT2 and decreased TEA accumulation in kidney slices from male, but not female, rats. Testosterone and estradiol treatment had no effect on rOCT1 mRNA or protein expression (Fig. 1). The authors suggest that OCT2 may have a physiological role in the secretion of endogenous substances. Other transporters may also play a role in the kidney transport of TEA.

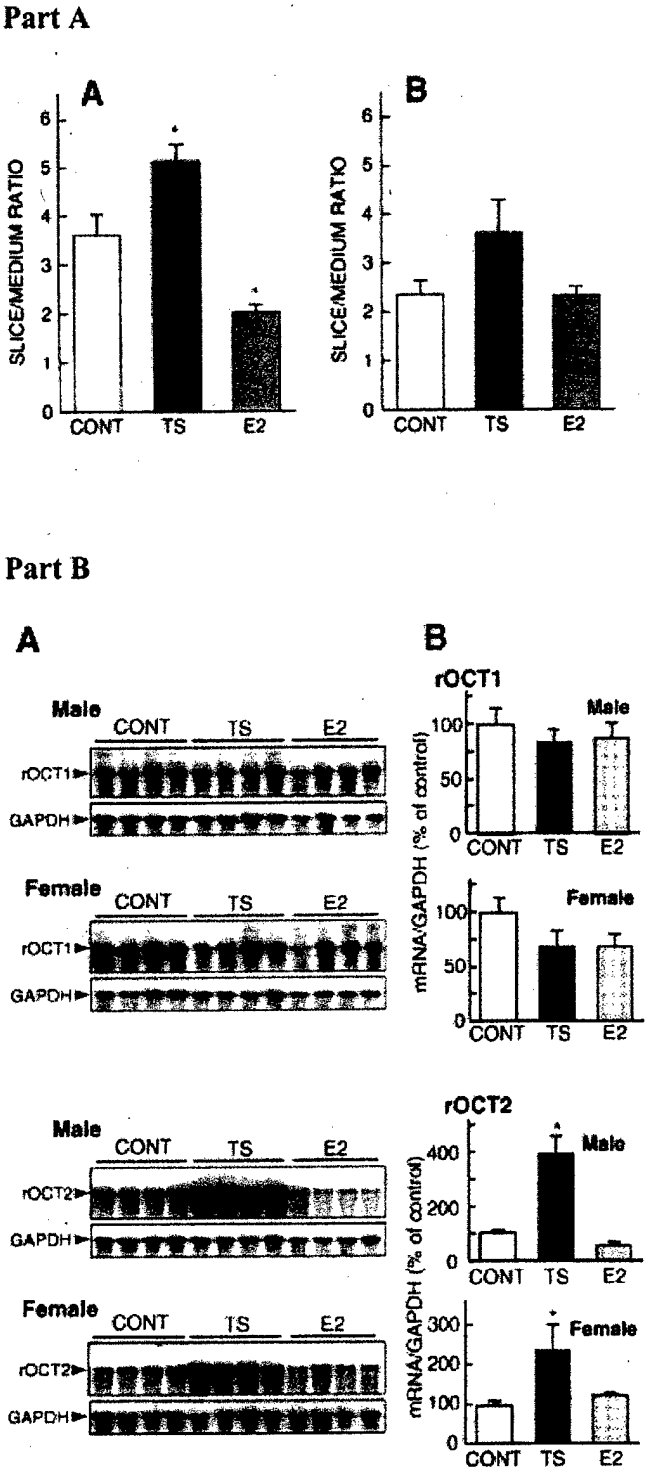


FIG. 1. TEA accumulation by kidney slices from male and female rats treated with testosterone and estradiol. Part A, kidney slices from males (A) and females (B) were incubated at 25°C in buffer containing 50 $\mu$M [$^{14}$C]TEA for 60 min. CONT, rats treated with vehicle; TS, rats treated with testosterone; E2, rats treated with 17$\beta$-estradiol. Each column represents the mean ± S.E. of three separate experiments. *, $p < 0.05$, significantly different from control. Part B, Northern blot analysis of total RNA of the kidney from male and female rats from CONT, TS, and E2 groups. Densitometric quantitation of rOCT1 and rOCT2 mRNA is corrected for loading using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each column represents the mean ± S.E. of four rats. *, $p < 0.05$. Reprinted with permission (Urakami et al., 2000).

P-glycoprotein (Pgp), present in the brush-border membrane of proximal tubule cells in the kidney, is involved in the renal elimination of a diverse range of lipophilic organic cations. Schinkel et al. (1994) reported a 1-fold higher expression of *mdr1b* in kidney isolated from female mice compared with male mice. The gene products of *mdr1a* and *mdr1b* (in mice) and MDR1 (in humans) are involved in xenobiotic transport and responsible for the multidrug resistance associated with Pgp overexpression in cancer cells. Potential gender differences in the kidney levels of Pgp in humans have not been examined; nor is there information regarding sex hormone effects on Pgp expression in the kidney. However, estrogen and progesterone may be important in the regulation of Pgp function; mRNA and protein expression for Pgp are greatly increased in the secretory luminal and glandular epithelium of the gravid murine uterus, suggesting regulation by the changes in estrogen/progesterone that occur in pregnancy (Arceci et al., 1990).

In clinical studies, the quinidine- and quinine-induced inhibition of renal amantadine clearance occurs only in healthy male subjects (Gaudry et al., 1993) and the urinary recovery at 48 h and the weight normalized renal clearance of amantadine are significantly higher in men than in women (Wong et al., 1995). The human organic cation transporters hOCT1, hOCT2, and hOCT3 have been cloned. hOCT2 is mainly expressed in the kidney but there is no information available regarding gender differences in expression (Dresser et al., 2001).

With regard to inorganic cations, the transepithelial calcium and magnesium reabsorption in the mouse cortical thick ascending limb of Henle's loop is greater in male than female animals, at both 4 and 8 weeks of age; there were no gender-related differences in NaCl transport (Wittner et al., 1997). There are sex differences in the uptake of inorganic mercury into kidney and motor neurons of mice. The uptake of mercury into the female kidney is much lower than that into the male kidney whereas inorganic mercury uptake by female motor neurons is 1.7 times greater than that in males. A smaller accumulation of mercury in the kidney of female mice may result in more circulating mercury which is available to enter muscle and taken up by distal motor axons (Pamphlett et al., 1997).

### *B. Liver*

*1. Anions.* Gender-associated differences in hepatic transport have been described for organic anions such as sulfobromophthalein (BSP), thymol blue, bilirubin, indocyanine green, tetrabromosulfonephthalein (TBS), and fatty acids. These organic anions are transported to a greater extent into hepatocytes isolated from the livers of female rats than male rats (Orzes et al., 1985; Sorrentino et al., 1988; Torres, 1996).

Marked differences have been reported for the hepatic uptake of a low concentration of BSP between male and female rats, both in intact animals and in isolated liver preparations and hepatocytes. The uptake rates of BSP in perfused livers, as well as the fractional plasma BSP disappearance rate, are significantly higher in females than in males. The kinetic constants of the low affinity sites are not different between genders whereas the $K_m$ of the high affinity uptake sites in females is significantly lower than that in males with no difference in $V_{max}$, suggesting that this may be due to a different structural arrangement of the transporter or to a different membrane environment at the sinusoidal domain (Orzes et al., 1985). TBS liver uptake rate in vivo, as well as in sinusoidal liver membrane vesicles, is greater in female rats. $V_{max}$ values for TBS uptake in the membrane vesicles are similar between male and female rats while $K_m$ values in males are significantly higher than that in females (5.5 ± 0.4 versus 17 ± 4 μM) (Fig. 2), suggesting that a difference in membrane transport rates may explain the greater accumulation or uptake of TBS in female hepatocytes (Torres, 1996).

Uptake of the glutathione conjugate of sulfobromophthalein (BSP-GSH) at steady state in single-pass liver perfusion studies is increased in female livers compared with male livers. The apparent $V_{max}$ is 48% larger in females whereas the apparent $K_m$ is similar in both sexes. The ratio of influx to efflux, which determines the equilibrium partition of BSP-GSH between the hepatocyte cytosol and plasma compartments, is significantly greater in females with no sex difference in the rate constant of biliary excretion. It has been suggested that these findings indicate that a less negative plasma membrane electrical potential in female livers may provide a more favorable electrochemical driving force for the

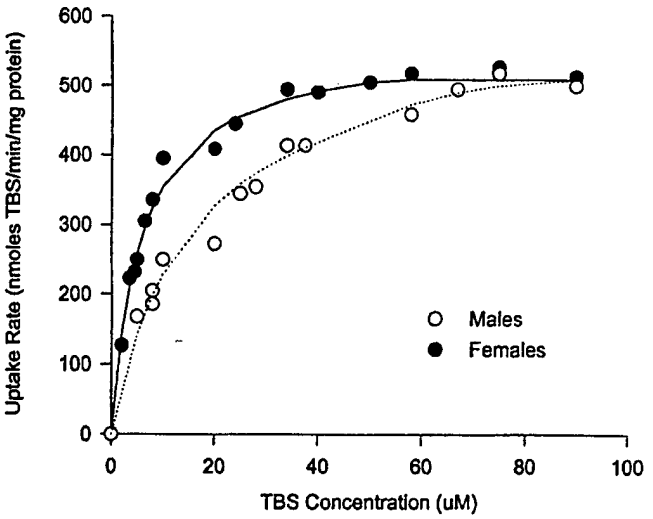


FIG. 2. Kinetics of TBS uptake in sinusoidal liver plasma membrane vesicles from male and female rats. Both curves represent the result of a typical experiment. $V_{max}$ values for TBS uptake are comparable for male and female rats (581 ± 60 versus 544 ± 15 nmol/min/mg of protein, mean ± S.D., $n = 3$); however, the $K_m$ values for TBS uptake in males are significantly higher than in females (17 ± 4.0 versus 5.5 ± 0.4 μM, mean ± S.D., $n = 3$, $p < 0.05$). Adapted from Torres, 1996 with permission from Elsevier Science.

movement of BSP-GSH into the hepatocytes in females (Sorrentino et al., 1988).

Initial oleate uptake velocity in hepatocytes isolated from female rats is also significantly greater than that from male rats. This may be due to a greater affinity of the transport system for oleate in females since no differences are observed in the $V_{max}$ value for hepatic oleate uptake as well as in the surface expression of plasma membrane fatty acid binding proteins between sexes (Sorrentino et al., 1992). Another fatty acid, palmitate, also exhibits a 2-fold higher steady-state uptake rate in livers of female rats compared with male rats (Luxon et al., 1998). Sex differences in the clearance of palmitate by human hepatocytes have been reported (Pond et al., 1996), with hepatocytes isolated from females exhibiting a 2-fold higher clearance.

Although many organic anions are transported to a greater extent by female hepatocytes, sodium-dependent taurocholate uptake is greater in male rats with a significantly higher $V_{max}$ value reported (Simon et al., 1999). Hepatic uptake of taurocholate, the major bile acid, is mainly mediated by the sodium-dependent taurocholate transporter (Ntcp) and to a lesser extent by Oatp. The initial uptake of sodium-dependent taurocholate uptake is shown over a range of concentrations (Fig. 3A). At every concentration, taurocholate uptake was greater in male hepatocytes. Suggested mechanisms that underlie the increased taurocholate transport in male hepatocytes are the greater expression of Ntcp (2-fold greater for both mRNA and protein levels) and the increased sinusoidal membrane fluidity (Lu et al., 1996). Simon et al. (1999) found that Ntcp, but not Oatp, protein content was significantly greater in males and that the expression of Ntcp was transcriptionally regulated. Hepatic Ntcp mRNA levels from female rats were 54 ± 4% of the value in males (Simon et al., 1999) (Fig. 3B). Female sinusoidal membranes had decreased fluidity (motional order) compared with male membranes, although bile canalicular membranes were not different. Liver sinusoidal membranes isolated from female rats exhibited changes in their phospholipid/fatty acid composition, in that they had a significantly increased phosphatidylethanolamine-to-phosphatidylcholine ratio. This decreased membrane fluidity in female hepatocytes may be involved in lower hepatic taurocholate uptake in females (Simon et al., 1999).

The reduction in the hepatic transport of rifamycin SV is more pronounced in male patients than in female patients with Gilbert's syndrome. This more pronounced defect in hepatobiliary transport in male subjects may explain, at least in part, the greater frequency of Gilbert's syndrome, a pathological condition characterized by unconjugated hyperbilirubinemia, in males (Gentile et al., 1985).

Gender-related differences in the biliary excretion of the organic anion tartrazine, a food dye, has been reported in the rat (Bertagni et al., 1972). Male and female

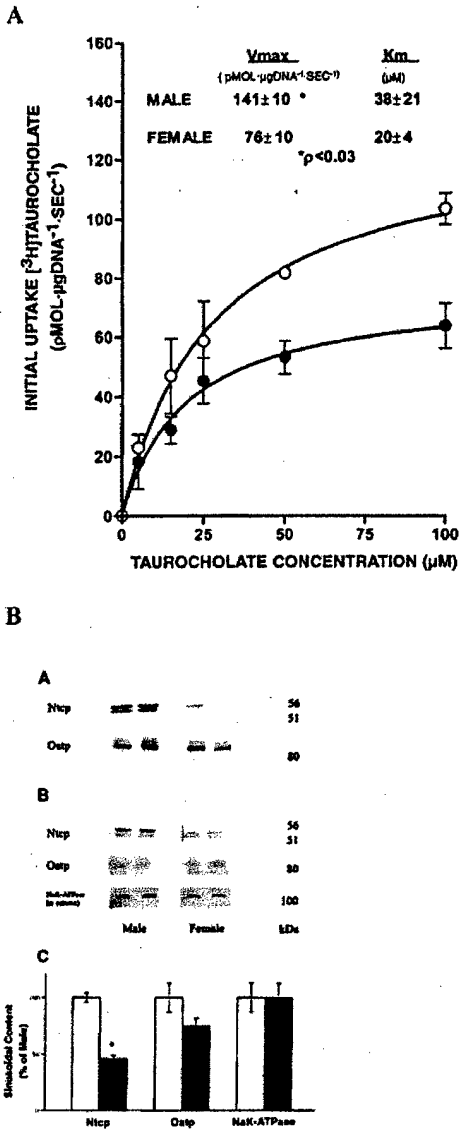


FIG. 3. Saturation kinetics for sodium-dependent taurocholate uptake into isolated rat hepatocytes. A, hepatocytes were isolated from male (○) and oophorectomized (ovx) female (●) rats, and initial uptake of [$^3$H]taurocholate was measured at 60 s in the presence and absence of sodium. These data were used to estimate the maximal uptake ($V_{max}$) and the Michaelis-Menten constant ($K_m$) values. Results are means ± S.E. of three independent experiments in each group. B, immunoblot analysis of sinusoidal membrane proteins from liver homogenates (A) and liver sinusoidal membrane fractions (B), from male and female rats. Liver sinusoidal proteins were identified using specific antibodies. Male (□) and female (■) hepatic steady-state levels of Ntcp, Oatp, and $Na^+$-$K^+$-ATPase are shown in panel C. Male levels were set at 100%. Results are mean ± S.E. of 4 to 12 separate determinations. *, $p < 0.01$. Reprinted with permission (Simon et al., 1999).

rats excrete 13 and 29%, respectively, of an intravenous dose of tartrazine by biliary excretion. Treatment of male rats with estradiol increased the excretion from 14 to 33% of the dose, although treatment of female rats with testosterone decreased the biliary excretion from 31 to 16%. Gender-related differences in the biliary excretion of *S*-ketoprofen have also been reported (Palylyk and Jamali, 1994). In male rats, the major route of elimination is by biliary excretion of the glucuronide

conjugate, whereas in the female rat, the major route of elimination is renal clearance of the conjugate. This results in a marked difference in the amount of *S*-ketoprofen glucuronide eliminated in the urine in female and male rats. There are gender differences in the ATP-dependent canalicular transport of dinitrophenyl-glutathione conjugate (Srivastava et al., 1999). Transport is higher in membrane vesicles isolated from male mice compared with female mice. Additionally, whereas only one transport system is present in male mouse cLPM for the transport of dinitrophenyl-glutathione, there is both high and low affinity systems present in cLPM isolated from female mice. The ATP-dependent transport of organic anions, including glucuronide and glutathione conjugates, occurs by multidrug resistance-associated protein 2 (MRP2), also known as the canalicular multispecific organic anion transporter (cMOAT). MRP2 is the major transporter responsible for secretion of bilirubin glucuronides into bile; gender differences in the expression of MRP2 have not been examined.

*2. Cations.* Pgp is present on the canalicular membrane of hepatocytes and involved in the biliary excretion of phospholipids, cholesterol, and a wide variety of lipophilic organic cations. Hepatic expression of the gene product of *mdr2* in female rats is 7-fold higher than in male rats (Furuya et al., 1994). This isoform of Pgp is mainly involved in phospholipid transport across the canalicular membrane. Piquette-Miller et al. (1998) and Salphati and Benet (1998) reported higher levels of total *mdr* gene products in female rat livers compared with male livers. Gender differences in *mdr* mRNA levels were also seen. Male livers contained more than 2-fold higher levels of *mdr1b* and female livers contained higher levels (approximately 35–50%) of *mdr1a* and *mdr2* (Piquette-Miller et al., 1998; Salphati and Benet, 1998). In humans, hepatic Pgp (total) protein expression is 2-fold higher in men than in women (Schuetz et al., 1995), suggesting that the drug disposition of Pgp substrates could be different between genders, resulting in differences in drug efficacy and toxicity between males and females. Interestingly, there are gender-related differences in Pgp expression and functional activity in peripheral blood samples of subjects with B-type chronic lymphocytic leukemia, with significantly more men (89%) than women (48%) being MDR1 phenotype-positive (Steiner et al., 1998). These findings are consistent with the overall better prognosis for women with chronic lymphocytic leukemia than for men (Steiner et al., 1998).

### C. Intestine

Very little is known regarding gender-related differences in intestinal uptake and drug bioavailability. Clinical studies have reported an increased bioavailability of both iron and ethanol in women but these gender-related differences likely do not involve differences in intestinal transporters. For ethanol, the increased bioavailability is likely due to decreased gastric metabolism of alcohol in women (Lieber et al., 1994). Decreases in both the rate and extent of absorption of acetaminophen occur in late pregnancy; this is likely due to decreases in the rate of gastric emptying (Galinsky and Levy, 1984).

A gender-related difference has been documented in the transport of calcium in the intestine. Kinetic analysis of calcium transport across the rat intestine has shown that there are two transport processes, one of which is saturable and the other nonsaturable. The saturable transport process is regulated by vitamin D and is predominantly located in the proximal intestine whereas the nonsaturable process is not vitamin D-dependent and has similar capacity throughout the intestine (Bronner et al., 1986). Intestinal calcium transport is significantly greater in male rats than in female rats in a vitamin D-sufficient condition, although it is comparable between sexes in the presence of vitamin D deficiency. Vitamin D deficiency produces a markedly lower intestinal transport of calcium in male rats but not in female rats. This observation suggests that calcium transport in the intestine of female rats, unlike male rats, is mediated by a vitamin D-independent mechanism at the calcium intake levels studied in this investigation (Uhland-Smith and DeLuca, 1993).

Total intestinal absorption of calcium is enhanced during pregnancy and lactation in vitamin D-deficient rats. Intestinal calcium absorption during the rat estrous cycle is highest during estrus and lowest during diestrus following the administration of both high and low calcium diets. Since the highest serum levels of estradiol, progesterone, prolactin, follicle-stimulating hormone, and luteinizing hormone are present during estrus (Butcher et al., 1974; Brommage et al., 1990), the greatest intestinal absorption of calcium observed during estrus may be related, either directly or indirectly, to any one of several sex hormones (Brommage et al., 1993). Intestinal mucosal cells contain estrogen receptors, and calcium uptake in duodenal cells is significantly enhanced by about 60% by 17$\beta$-estradiol at a concentration of 10 nM (Arjmandi et al., 1993). Administration of 17$\beta$-estradiol at a dose of 40 $\mu$g/kg b.wt./day for 21 days significantly elevated intestinal absorption of calcium in female rats whereas serum levels of 1,25-dihydroxyvitamin D were unaltered (Arjmandi et al., 1994). These findings suggest that transluminal calcium uptake is promoted by a direct action of 17$\beta$-estradiol on the intestinal tract with no increase in the circulating levels of 1,25-dihydroxyvitamin D (Arjmandi et al., 1993, 1994).

The implications of these observations are as follows. First, estrogen may play an important physiological role in regulation of intestinal calcium absorption. High estrogen levels during pregnancy and estrus may promote calcium absorption, and estrogen deficiency in menopause may result in calcium malabsorption by a direct action on the intestine. The malabsorption of calcium in

the intestine as a result of ovarian hormone deficiency in postmenopausal women is often associated with osteoporosis characterized by bone loss (Heaney et al., 1978; Gallagher et al., 1979, 1980; Gallagher, 1990). Second, the rate and extent of intestinal calcium absorption may be modulated by compounds that block or mimic estrogen action.

Aluminum, at a concentration of 2 $\mu$M, significantly decreases mucosa-to-serosa calcium influx in duodenal everted sacs both of male and female rats compared with aluminum-free controls; however, the percentage of reduction in females (31.2%) is greater than that in males (17.8%). The sensitivity to the inhibitory effect of aluminum on duodenal calcium flux is raised with increasing serum levels of 17$\beta$-estradiol in ovariectomized female rats with no alterations in the maximal response, whereas the effect of aluminum on calcium flux in duodenal sacs is not dependent of serum testosterone levels in castrated male rats injected with testosterone. These results demonstrate that there are gender-associated differences in the inhibitory effect of aluminum on transluminal calcium transport in the duodenum of the rat (Orihuela et al., 1996).

*D. Brain*

There have been few studies that have examined the potential for gender-related differences in transport across the blood-brain barrier (BBB). 17$\beta$-Estradiol treatment of ovariectomized rats increases 2-deoxyglucose uptake into brain, which is likely due to the increase in the mRNA and protein expression of glucose transporter 1 (GLUT-1) in the BBB epithelium (Shi and Simpkins, 1997). These results support a modulatory role for estrogens in the brain transport of glucose.

Both Pgp and MRP1 are present in the BBB epithelium and are responsible for the active efflux of drugs from the brain, minimizing brain exposure to many organic anions and cations. Although Pgp exhibits gender differences in expression in liver, this has not been examined for the BBB. Gender differences in the BBB uptake of verapamil have been reported in mice where female mice have increased functional Pgp activity, resulting in decreased verapamil influx into the brain (Dagenais et al., 2001). However, gender differences in uptake were not observed for two other Pgp substrates, morphine or quinidine (Dagenais et al., 2001), so the significance of these findings is unknown.

The effect of gender on the reuptake of dopamine (DA) by the sodium-dependent DA transporter into nerve terminals, the primary mechanism for inactivation of DA following its release into the synapse, has been examined. An increased synaptosomal DA reuptake in the anterior hypothalamus is observed in ovariectomized rats treated with estradiol due to an increase in the number of DA uptake binding sites (Cardinali and Gomez, 1977). The maximal binding density ($B_{max}$) of striatal DA uptake sites is significantly elevated 15 and 30 min after an injection of a physiological dose of 17$\beta$-estradiol in ovariectomized rats with no change in the binding affinity ($K_d$) of the DA uptake sites. There is no effect of progesterone on striatal DA uptake after progesterone treatment of ovariectomized rats. The increase of DA uptake binding sites by the administration of 17$\beta$-estradiol is rapid and short-lasting and is associated with peak 17$\beta$-estradiol plasma levels, suggesting most likely a membrane-linked nongenomic effect of 17$\beta$-estradiol (Morissette et al., 1990). When ovariectomized rats are chronically treated with 17$\beta$-estradiol and/or progesterone at pharmacological doses, DA uptake site density in the striatum is significantly increased by 16 to 23% without an alteration in the binding affinity, most likely due to an increased synthesis of the DA transporter by a genomic effect of these female sex hormones. In addition, chronic exposure to 17$\beta$-estradiol and/or progesterone up-regulates the DA uptake sites in the nigrostriatal dopaminergic pathway whereas the nucleus accumbens and the substantia nigra pars reticula are not affected (Morissette and Di Paolo, 1993a). Striatal DA uptake site density is significantly lower in normal male rats, gonadectomized male rats, and ovariectomized female rats compared with normal female rats and fluctuates during the female estrous cycle with a peak occurring in the morning of proestrus when estradiol is elevated and progesterone is low, suggesting an up-regulation of striatal DA uptake sites by estradiol (Morissette and Di Paolo, 1993b). This agrees with the findings of an investigation examining the effect of acute treatment with 17$\beta$-estradiol (Morissette et al., 1990). It has also been shown that 17$\beta$-estradiol increases DA uptake in mesencephalic neurons isolated from females but not in male neurons, and male sex hormones, testosterone and dihydrotestosterone, have no effect (Engele et al., 1989). In humans, DA and serotonin (5-hydroxytryptamine; 5-HT) transporter availability is greater in females compared with males, as determined by single photon emission computed tomography imaging using an analog of cocaine (CIT) that labels DA and 5-HT transporters (Staley et al., 2001). Therefore, gonadal hormones may play an important role in the effects of psychoactive drugs acting on neuronal DA uptake sites and modulation of the DA transporter by these hormones will represent a source of interindividual variability in the treatment of neuropsychiatric disorders and neurologic diseases such as Parkinson's disease (Cardinali and Gomez, 1977; Engele et al., 1989; Morissette et al., 1990; Morissette and Di Paolo, 1993a,b).

A sexual dimorphism in the density of norepinephrine transporters has been demonstrated in the frontal cortex of rats, with males having significantly fewer binding sites than females, whereas the binding affinity of the uptake sites was not different between genders (Vathy et al., 1997). 5-HT uptake in the anterior and middle hypothalamus of intact female rats exceeds sig-

nificantly that in intact male rats (by about 30–40%) and is similar to that in neonatally castrated adult rats (Fig. 4), suggesting that androgens may play a key role in the development of the hypothalamic serotoninergic system over the neonatal period by inhibiting either the serotoninergic axon ingrowth to the hypothalamus or the ramifications of the axonal terminal portions (Borisova et al., 1996). Estradiol treatment stimulates a significant increase in the density of 5-$HT_{2A}$ binding sites in the anterior frontal, anterior cingulate and piriform cortex, the olfactory tubercle, the nucleus accumbens and the lateral dorsal raphe nucleus, areas of brain concerned with cognition, emotion, and motor control, suggesting that the antidepressant action of estrogen may be mediated by a serotoninergic mechanism (Fink et al., 1996).

### *E. Other Tissues*

The rate of glucose uptake in skeletal muscle, under hyperinsulinemic and normoglycemic conditions, is significantly greater in women than in men (Fig. 5), suggesting an increased sensitivity to insulin in women (Nuutila et al., 1995). Basal and maximal insulin-stimulated glucose transport is also significantly higher in adipocytes isolated from female rats and human female subjects compared with males (Foley et al., 1984). In skeletal muscle, fatty acid transport protein-1 (FATP-1) mRNA levels are higher in lean women than in lean men ($2.2 \pm 0.1$ versus $0.6 \pm 0.2$ attomoles/$\mu$g of total RNA, $p < 0.01$). FATP-1 mRNA was significantly decreased in skeletal muscle of obese women, but no change in FATP-1 expression was seen in men. Additionally, insulin infusion reduced FATP-1 mRNA in muscle of lean women, but not in men (Binnert et al., 2000). This study indicates that lean women may be able to utilize lipids to a greater extent than men, although whether differences in FATP-1 mRNA result in corresponding differences in FATP-1 protein expression is not known.

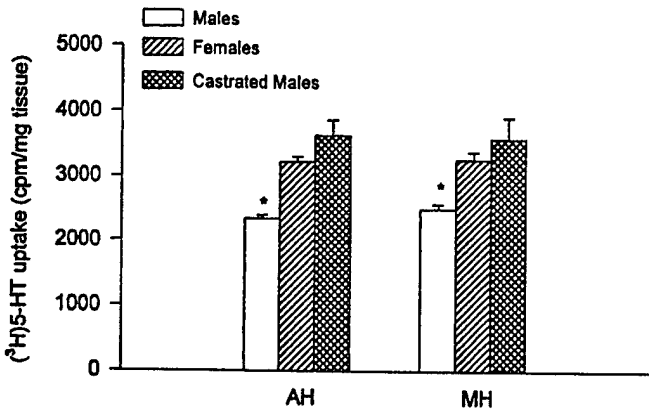


FIG. 4. Specific uptake of radioactively labeled serotonin ([$^3$H]5-HT) by the anterior (AH) and middle hypothalamus (MH) in adult male, adult female, and neonatally castrated males. Specific uptake is the difference of [$^3$H]5-HT uptake in the absence and presence of citalopram $10^{-5}$ M. The columns represent the values for 10 to 25 rats. *, $p < 0.001$ compared with the levels in females and castrated males. Adapted with permission from Borisova et al., 1996.

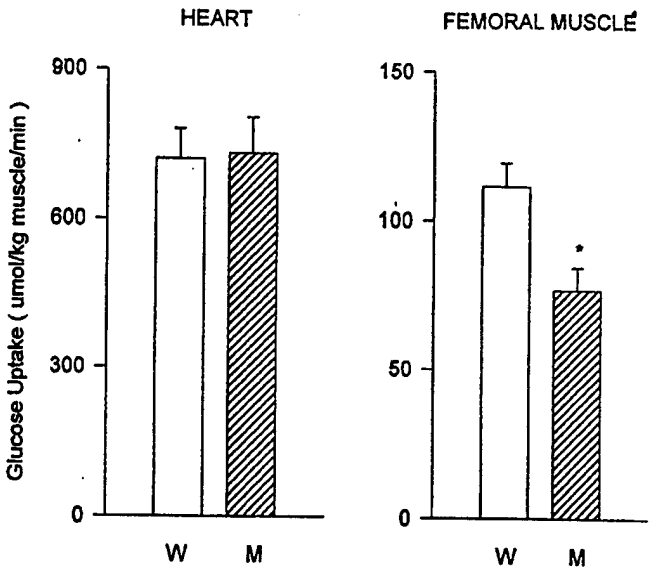


FIG. 5. Rates of glucose uptake in the heart and femoral muscles (micromoles per kilogram of muscle per minute) in normal women (W) and men (M). Insulin sensitivity of glucose uptake was determined in heart and muscle tissues using positron emission tomography under hyperinsulinemic and normoglycemic conditions. *, $p < 0.01$ compared with women. Adapted with permission from Nuutila et al., 1995.

A marked difference in the splanchnic uptake of chylomicron triglyceride is observed between men and women. Chylomicron uptake in the splanchnic tissues in men and women accounts for 71% and 20% of meal triglyceride disposal, respectively, indicating greater meal fatty acid storage in visceral adipose tissue in men and gender-specific differences in body fat distribution (Nguyen et al., 1996).

## III. Conclusions

Gender differences in the transport of numerous drugs and endogenous substrates exist in animals and humans. Sex-associated differences are described for renal tubular secretion of organic anions and cations, hepatic uptake of taurocholate and organic anions including endogenous compounds, intestinal calcium transport, and Pgp-mediated and neurotransmitter transport in the brain. Gender-related differences in transporter mRNA and protein expression represent an important mechanism for the regulation of hepatic transport processes. Furthermore, female sex hormones, mainly estradiol, and male sex hormones, primarily testosterone, appear to be involved in these gender-related differences in transport either directly or indirectly. In addition, gonadal hormones can be used to treat neurologic diseases and neuropsychiatric disorders by modulating the DA uptake sites in the brain.

Gender differences in membrane transport in humans are not always consistent with differences reported in animal studies. For example, Pgp, an ATP-dependent efflux pump present in cancer cells and excretory or-

gans, demonstrates a higher hepatic expression in men than in women (Schuetz et al., 1995), but opposite changes have been reported in rats (Furuya et al., 1994; Piquette-Miller et al., 1998). In addition, gender differences in the urinary excretion of zenarestat are observed in mice and rats but not in dogs and humans (Tanaka et al., 1992). This emphasizes the importance of performing studies in humans to evaluate the effect of gender.

Gender-associated differences in the nature and prevalence of many diseases may be explained, at least in part, by the differences in the transport processes of substrates between male and female subjects. In addition, these gender-related differences in transport systems may be responsible, at least in part, for interindividual variability in drug disposition, therapeutic response, and drug toxicity. Research is needed to evaluate potential gender differences in regulation, expression, and activity of known transport proteins involved in the uptake or secretion of both endogenous and exogenous compounds.

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# Determination of α-naphthylisothiocyanate and metabolites α-naphthylamine and α-naphthylisocyanate in rat plasma and urine by high-performance liquid chromatography

Ke Hu, Marilyn E. Morris*

*Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, 517 Hochstetter Hall, State University of New York at Buffalo, Amherst, NY 14260, USA*

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**Abstract**

A rapid and sensitive high-performance liquid chromatographic (HPLC) assay for the determination of α-naphthylisothiocyanate (1-NITC) and two metabolites α-naphthylamine (1-NA) and α-naphthylisocyanate (1-NIC) in rat plasma and urine has been developed. The chromatographic analysis was carried out using reversed-phase isocratic elution with a Partisphere $C_{18}$ 5-μm column, a mobile phase of acetonitrile–water (ACN–$H_2O$ 70:30, v/v), and detection by ultraviolet (UV) absorption at 305 nm. The lower limits of quantitation (LLQ) in rat plasma, urine, and ACN were 10, 30, and 10 ng/ml for 1-NITC; 30, 100, and 30 ng/ml for 1-NA; and 30 ng/ml in ACN for 1-NIC. At low (10 ng/ml), medium (500 ng/ml), and high (5000 ng/ml) concentrations of quality control samples (QCs), the range of within-day and between-day accuracies were 95–106 and 97–103% for 1-NITC in plasma, respectively. Stability studies showed that 1-NITC was stable at all tested temperatures in ACN, and at −20 and −80 °C in plasma, urine, and ACN precipitated plasma and urine, but degraded at room temperature and 4 °C. 1-NA was stable in all of the tested matrices at all temperatures. 1-NIC was unstable in plasma, urine, and ACN precipitated plasma and urine, but stable in ACN. The degradation product of 1-NITC and 1-NIC in universal buffer was confirmed to be 1-NA. 1-NITC and 1-NA were detected and quantified in rat plasma and urine, following the administration of a 25 mg/kg i.v. dose of 1-NITC to a female Sprague–Dawley rat.


*Keywords:* α-Naphthylisothiocyanate; α-Naphthylamine; α-Naphthylisocyanate

## 1. Introduction

Many synthetic and naturally occurring organic isothiocyanates (ITCs; RN=C=S) can block chemical carcinogenesis in experimental animals and are being considered as chemopreventive agents for human use (see reviews in Refs. [1,2]). α-Naphthylisothiocyanate (1-NITC) (Fig. 1) was reported as a carcinogenesis inhibitor in rats as early as the 1960s [3–7]. Recently, we have found that 1-NITC can reverse the multidrug resistance (MDR) to antineoplastic agents in human cancer cell lines through inhibition of the ATP-dependent efflux proteins, P-glycoprotein and multidrug resistance associated-protein 1 (MRP1) [8]. These findings indicated the potential use of 1-NITC not only in cancer preven-

*Corresponding author. Tel.: +1-716-645-2842x230; fax: +1-716-645-3693.
*E-mail address:* memorris@acsu.buffalo.edu (M.E. Morris).

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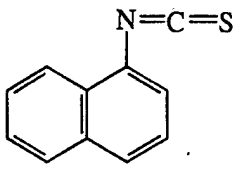
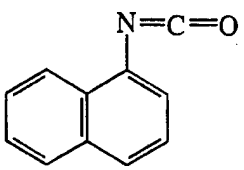



1-NITC

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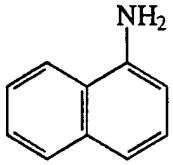
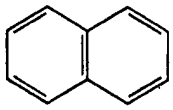



1-NA



NE

Fig. 1. Chemical structures of 1-NITC, 1-NA, 1-NIC and NE.

tion but as a chemosensitizing agent. High doses of 1-NITC (150–300 mg/kg) produce hepatic lesions resembling those occurring with biliary cirrhosis in humans [9–11]; this has led to the use of 1-NITC as a model chemical to study human cirrhosis and drug-induced cholestasis.

To our knowledge, the pharmacokinetics of 1-NITC and its metabolites in rat plasma and urine have not been studied, although it is reported that 1-NITC is eliminated in the bile and urine predominantly as metabolites [12]. The major metabolites of 1-NITC have been characterized by mass spectroscopy and found to be α-naphthylisocyanate (1-NIC) and α-naphthylamine (1-NA) [16]. Several analytical methods have been described for the determination of 1-NITC in biological samples, including assays based on radiolabelled drug [10–13], thin-layer chromatography [14], gas–liquid chromatography (GLC) [15], gas chromatography/mass spectrometry (GC–MS) [16], and high-performance liquid chromatography (HPLC) [17–22]. However, those methods were labor intensive, time consuming, inconvenient, or not sensitive enough. In this paper we report the development and validation of a rapid and sensitive HPLC assay able to detect the parent drug 1-NITC and its metabolites in rat plasma and urine, the stability of 1-NITC and its metabolites in rat plasma and urine samples, and the use of the assay to characterize the pharmacokinetics of 1-NITC in a preliminary rat study.

## 2. Experimental

### 2.1. Chemicals and reagents

1-NITC and 1-NA were purchased from Sigma (St. Louis, MO, USA) more than 99 and 98% purity, respectively. 1-NIC was purchased from Aldrich (Milkwaukee, MI, USA) at 98% purity. The internal standard naphthalene (NE) (Fig. 1) was purchased from Fisher Scientific (Fair Lawn, NJ, USA) at more than 99% purity. Acetonitrile (ACN) and methanol (MeOH) were HPLC grade from Fisher. Other chemicals are in analytical grade unless specified.

### 2.2. Preparation of rat plasma and urine samples for calibration of standards and quality control samples (QCs)

The stock solutions 10 mg/ml of 1-NITC, 1-NA, 1-NIC, and NE were freshly prepared for every validation run by dissolving a weighted amount of each compound in ACN. The 0.5 and 2.0 mg/ml working solutions of NE were prepared by diluting the stock solution with ACN as internal standard for validation of 1-NITC and 1-NA in rat plasma and urine samples, respectively.

Solutions of 1-NITC containing 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, and 250 μg/ml were prepared by serial dilution of the stock solutions with ACN. Each blank rat plasma sample (50 μl) was spiked with 5 μl of a NE solution (0.5 mg/ml), 5 μl of varying concentrations of 1-NITC, and 190 μl ACN, to prepare a series of standards (10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/ml as final concentration) for the calibration curve.

The working solutions of 1-NA containing 5, 10, 25, 50, 100, 250, 500, 1000, and 2500 μg/ml were prepared by serial dilution of the stock solutions with ACN. Each blank rat urine sample (50 μl) was spiked with 5 μl NE work solution (2.0 mg/ml), 5 μl appropriate 1-NA working solution, and added 190 μl ACN, to prepare a series of standards (100,

200, 500, 1000, 2000, 5000, 10 000, 20 000, and 50 000 ng/ml as final concentration) for the calibration curve.

Both spiked plasma and urine samples were vortexed for 10 s and centrifuged at 10 000 *g* for 5 min at 4 °C. The resulting supernatants were used for injection. QC samples at low (10 ng/ml for 1-NITC and 100 ng/ml for 1-NA), medium (500 ng/ml for 1-NITC and 5000 ng/ml for 1-NA), and high concentrations (5000 ng/ml for 1-NITC and 50 000 ng/ml for 1-NA), respectively, were prepared by the same procedures as previously described.

### 2.3. HPLC instrumentation and conditions

The Waters HPLC system (Milford, MA, USA) consisted of a model 1525 binary pump, a model 717plus autosampler (a 250-μl injector and a 200-μl loop) configured with a heater/cooler, a model 5HC column oven, and a model 2487 UV detector. The column and autosampler temperatures were kept at room temperature (21±1 °C) and 4 °C, respectively. The reversed-phase chromatography was performed with a Partisphere $C_{18}$ 5-μm column 125×4.6 mm I.D. (Whatman, Clifton, NJ, USA) protected by a RP guard cartridge system $C_{18}$ 5-μm (Whatman), and eluted isocratically with a mobile phase consisting of ACN–$H_2O$ (70:30, v/v). The flow-rate was 1.0 ml/ml and the injection volume was 50 μl. The UV detector was set at a single wavelength of 305 nm. The Breeze System software version 3.2 (Waters) was used for instrument control and data analysis.

### 2.4. Assay validation

#### 2.4.1. Lower limit of quantitation

The lower limit of quantification (LLQ) was determined during the evaluation of the linear range of calibration curve. LLQ was defined as the concentration of the lowest QC samples producing an assayed concentration within 10% of the theoretical value (i.e. accuracy between 90 and 110%) and yielding a precision of more than 90% for both within- and between-day evaluation.

#### 2.4.2. Linearity of calibration curve

The linearity of calibration curve was evaluated by regression analysis of peak area ratios (1-NITC/NE and 1-NA/NE) to 1-NITC and 1-NA concentrations in blank plasma and urine samples, respectively.

#### 2.4.3. Precision and accuracy

The assay was validated by within- and between-day accuracy and precision quantifying 1-NITC and 1-NA at QCs. Accuracy was determined by comparing the calculated concentration using calibration curves to known concentrations. Within-day variability was assessed through the analysis of QCs in triplicate, and between-day variability was determined through the analysis of QCs on four consecutive days.

#### 2.4.4. Recovery

The recovery of 1-NITC and 1-NA was established with QCs by comparing peak area ratios (1-NITC/NE and 1-NA/NE) to those of standards in ACN. The mean recoveries at low, medium, and high concentrations were determined for both within- and between-day analyses.

### 2.5. Stability

The stability of 1-NITC, 1-NA, and 1-NIC was studied in different matrices consisting of rat plasma, urine, ACN precipitated plasma and urine, ACN, and in a universal buffer (citrate–phosphate–borate–HCl, pH 2–12) at four designated temperatures over 96 h. 1-NITC, 1-NA, or 1-NIC (200 ng/ml as final concentration), along with internal standard NE (10 μg/ml), were added to plasma and ACN, respectively, for stability evaluations in plasma, ACN precipitated plasma, and ACN samples at room temperature (RT), 4, −20, and −80 °C. Samples were assayed at time points up to 96 h. The stability of 1-NITC, 1-NA, and 1-NIC in urine and ACN precipitated urine were tested at similar time intervals up to 96 h at a final concentration of 10 μg/ml for 1-NITC, 1-NA, and 1-NIC and 50 μg/ml for NE. The stabilities of 1-NITC, 1-NA, and 1-NIC in universal buffer were determined over a pH range from 2 to 12 at RT at times up to 96 h using the same concentrations as used for plasma samples. The compound was considered stable if the variation of

quantitation was less than 10% (i.e. 90–110% of initial time concentration).

### 2.6. 1-NITC pharmacokinetics in rat

The jugular vein cannula was inserted into a female Sprague–Dawley (Harlan, Indianapolis, IN, USA) rat following an i.m. injection of ketamine 90 mg/kg and xylazine 10 mg/kg (Henry Schein, Melville, NY, USA). Three days following surgery, a dose of 25 mg/kg 1-NITC (10 mg/ml) in a vehicle consisting of 10% ethanol (Pharmaco Products, Brookfield, CT, USA), 10% cremophor EL (Sigma), and 80% sterile saline (Braun Medical, Irvine, CA, USA) solution was administered as an intravenous (i.v.) bolus through the cannula.

Blood samples (250 μl each) were collected at 5, 10, 20, 30 min, 1, 2, 4, 6, 9, 12, and 24 h following 1-NITC administration, and placed in heparinized 0.6-ml microcentrifuge tubes. The plasma was immediately separated from blood via centrifugation at 1000 *g* for 10 min at 4 °C and stored at −80 °C to prevent potential degradation of 1-NITC and metabolites. The internal standard (5 μl) was added to 50 μl of each plasma sample and treated as previously described. The data was fitted to obtain pharmacokinetic (PK) parameters using WinNonLin version 2.1 (Pharsight, Mountain View, CA, USA).

Urine samples were collected at 2, 4, 6, 9, 12, 24, and 25 h time points, and the volume was measured. After adding 0.1% sodium azide (Fisher), the urine samples were centrifuged at 1000 *g* for 10 min at 4 °C and stored at −80 °C to prevent potential degradation of 1-NITC and 1-NA. Five μl NE (2.0 mg/ml) was added to 50 μl of each urine sample before assay.

## 3. Results

### 3.1. Specificity and selectivity

Figs. 2 and 3 display typical chromatograms resulting from HPLC analysis of the ACN precipitated rat plasma and urine. Blank rat plasma and urine do not demonstrate any interference peaks (Figs. 2a and 3a). The mixture of 1-NITC, 1-NA and 1-NIC (200 ng/ml each) and internal standard in ACN solution are well separated from one another with retention times ($t_R$) of 1-NA (2.2 min), NE (3.2 min), 1-NIC (3.7 min), and 1-NITC (5.6 min) (Fig. 2b). The rat plasma and urine samples spiked with 1-NITC, 1-NA, 1-NIC and NE standards show similar results (Figs. 2c and 3b), except that 1-NIC is absent due to possible rapid degradation in plasma and urine samples (Figs. 2d and 3c). 1-NITC, 1-NA, and NE are separated well from potentially interfering endogenous plasma and urine compounds under the current optimal chromatographic conditions (Figs. 2a,c,d, and 3a–c). In biological samples obtained after the i.v. administration of 1-NITC to a rat, 1-NITC and 1-NA were the only compounds that could be detected in plasma (Fig. 2e) and urine (Fig. 3d), respectively.

### 3.2. Lower limit of quantitation (LLQ)

The LLQ of 1-NITC, 1-NA, and 1-NIC was determined in blank rat plasma and urine samples, as well as in ACN solution. As shown in Table 1, the lower limit of quantitation (LLQ) of 1-NITC, 1-NA, and 1-NIC are dependent on the matrix. The LLQ of 1-NITC is 10 ng/ml for plasma and ACN samples, and 30 ng/ml for urine samples. The LLQ of 1-NA is about three-fold more than 1-NITC, i.e. 30 ng/ml for blank rat plasma and ACN, and 100 ng/ml for blank rat urine. 1-NIC can be detected only in ACN with a LLQ of 30 ng/ml.

### 3.3. Linearity

The linear regression correlation coefficient $r$ was more than 0.999 in every standard curve (data not shown). The linearity for 1-NITC and 1-NA was tested over a concentration range of 10–5000 ng/ml and 30–5000 ng/ml, respectively, in rat plasma. For rat urine samples, the calibration curves of 1-NITC and 1-NA were linear over the concentration range of 30–5000 and 100–50 000 ng/ml, respectively.

### 3.4. Accuracy, precision and recovery

As shown in Table 2, at low (10 ng/ml), medium (500 ng/ml), and high (5000 ng/ml) concentrations of 1-NITC, the within- and between-day accuracy were 95–106 and 97–103%, respectively. The with-

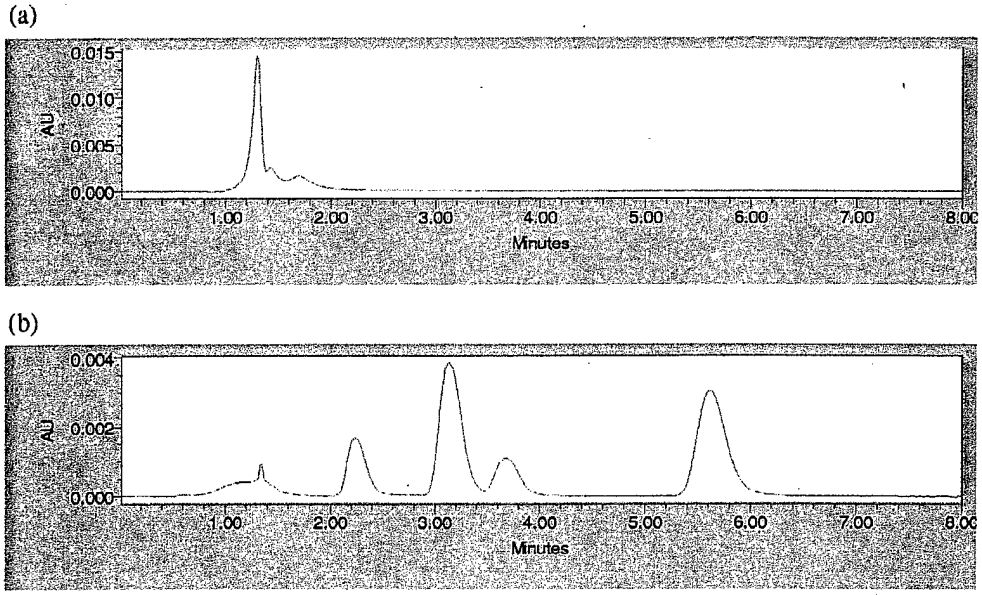



Fig. 2. Typical chromatograms for rat plasma samples obtained from the analysis of (a) blank plasma. (b) ACN containing 1-NITC (200 ng/ml), 1-NA (200 ng/ml), 1-NIC (200 ng/ml), and NE (10 μg/ml). (c) Blank plasma with added 1-NITC (200 ng/ml), 1-NA (200 ng/ml), 1-NIC (200 ng/ml), and NE (10 μg/ml), following protein precipitation with ACN; (d) blank plasma with added 1-NITC (200 ng/ml), 1-NA (200 ng/ml), and NE (10 μg/ml), following protein precipitation with ACN with the supernatant spiked with 1-NIC (200 ng/ml); (e) a 2-h rat plasma sample obtained after an i.v. bolus of 25 mg/kg 1-NITC. Chromatographic peaks were identified with the aid of pure reference standards based on retention time $t_R$, including 1-NA (2.2 min), NE (3.2 min), 1-NIC (3.7 min), and 1-NITC (5.4–5.9 min in different matrices).

in- and between-day precision values were 97–100 and 93–97%, respectively. Moreover, the protein precipitation with ACN for plasma samples resulted in the recovery of 1-NITC between 93 and 97% for both within- and between-day analysis.

At low (100 ng/ml), medium (5000 ng/ml), and high (50 000 ng/ml) concentrations of 1-NA, the within- and between-day accuracy was 96–106%, precision 97–99%, and recovery 95–110% (Table 3).

### 3.5. Stability

1-NITC was stable at temperatures of −20 °C and −80 °C in plasma, urine, ACN precipitated plasma and urine (Fig. 4a–d), and at all tested temperatures in ACN over 96 h (data not shown). However, 1-NITC degraded at RT and 4 °C in plasma, urine, and ACN precipitated plasma and urine (Fig. 4a–d). The faster degradation at RT than at 4 °C indicated a temperature-dependent pattern in each matrix (Fig. 4a–d). Moreover, the degradation of 1-NITC in plasma (Fig. 4a) and urine (Fig. 4c) was greater than that in ACN precipitated plasma (Fig. 4b) and urine (Fig. 4d) at same temperatures (RT and 4 °C). The degradation of 1-NITC in ACN diluted urine (Fig. 4d) was much slower than ACN precipitated plasma (Fig. 4b); 1-NITC was stable when prepared in ACN at all temperatures (Fig. 4e). 1-NITC degraded with very similar patterns over the pH range of 2–10 over a 96-h period (Fig. 4f). A different pattern of degradation was observed at pH 11 (Fig. 4f); at pH 12 there was instantaneous degradation (data not shown). The degradation product of 1-NITC in universal buffer was confirmed to be 1-NA (data not shown). The degradation product of 1-NITC in plasma, urine, and ACN extracts of plasma and urine was not identified.

1-NA was stable in all matrices at RT, 4, −20, and −80 °C with quantitation variation less than 10% during individual test periods (plasma data only is shown in Fig. 4g); it was also stable over the pH

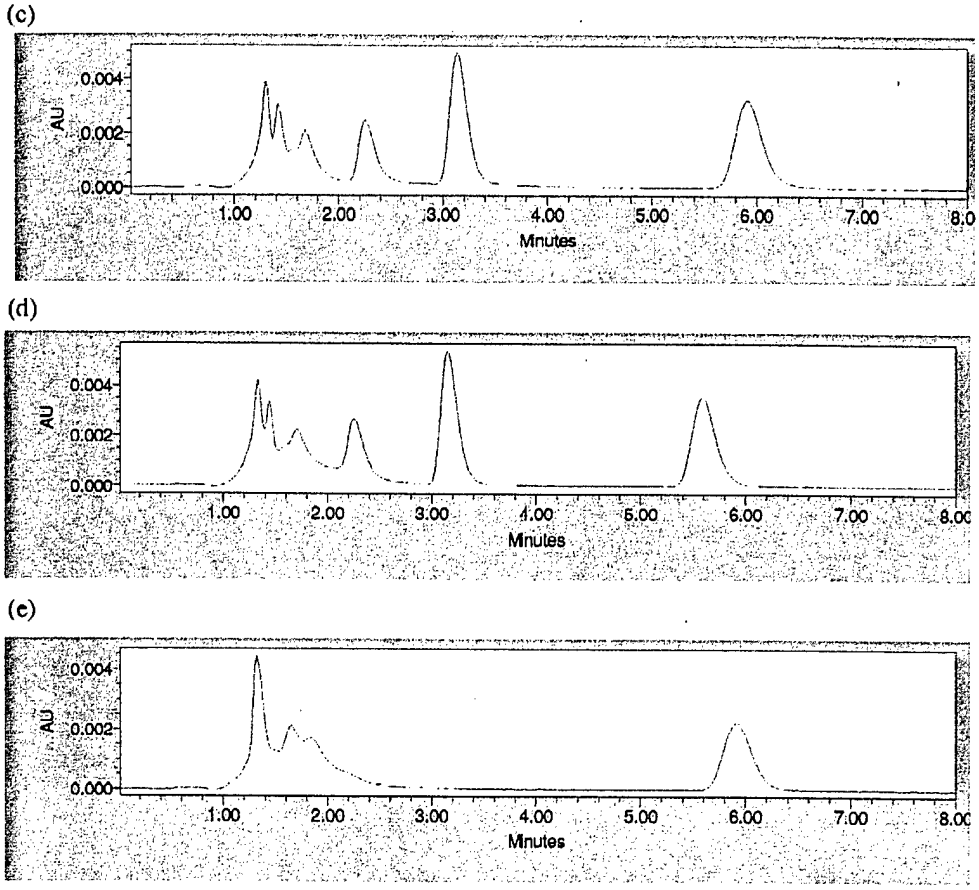



Fig. 2. (*continued*)

range of 2–12 (data not shown). In comparison, 1-NIC was stable when prepared in ACN (data not shown) but rapidly degraded in plasma (Fig. 2d), urine (Fig. 3c) and in ACN precipitated plasma and urine (data not shown). In universal buffer, 1-NIC was rapidly degraded to form 1-NA (data not shown).

### *3.6. Application of assay in rat pharmacokinetic studies*

The described analytical method was used to analyze plasma and urine samples following the administration of 1-NITC (25 mg/kg i.v.) to a rat. The parent drug 1-NITC and metabolite 1-NA were the only compounds that could be detected in plasma and urine samples, respectively (Figs. 2e and 3d). The concentration of 1-NITC in plasma over 24 h and 1-NA in urine over 25 h are given in Tables 4 and 5 and plasma data are plotted in Fig. 5. Using this HPLC assay, 1-NITC and 1-NA were quantified in rat plasma and urine, respectively (Tables 4 and 5). Analysis of plasma samples allowed the determination of the pharmacokinetic parameters for 1-NITC (clearance of 2.07 l/kg/h, apparent volume of distribution of 14.3 l/kg, and elimination half life of 4.76 h). The metabolite 1-NA was present in urine samples but the total recovery was about 0.4%.

## 4. Discussion

A rapid and sensitive high-performance liquid chromatographic (HPLC) assay for the determination

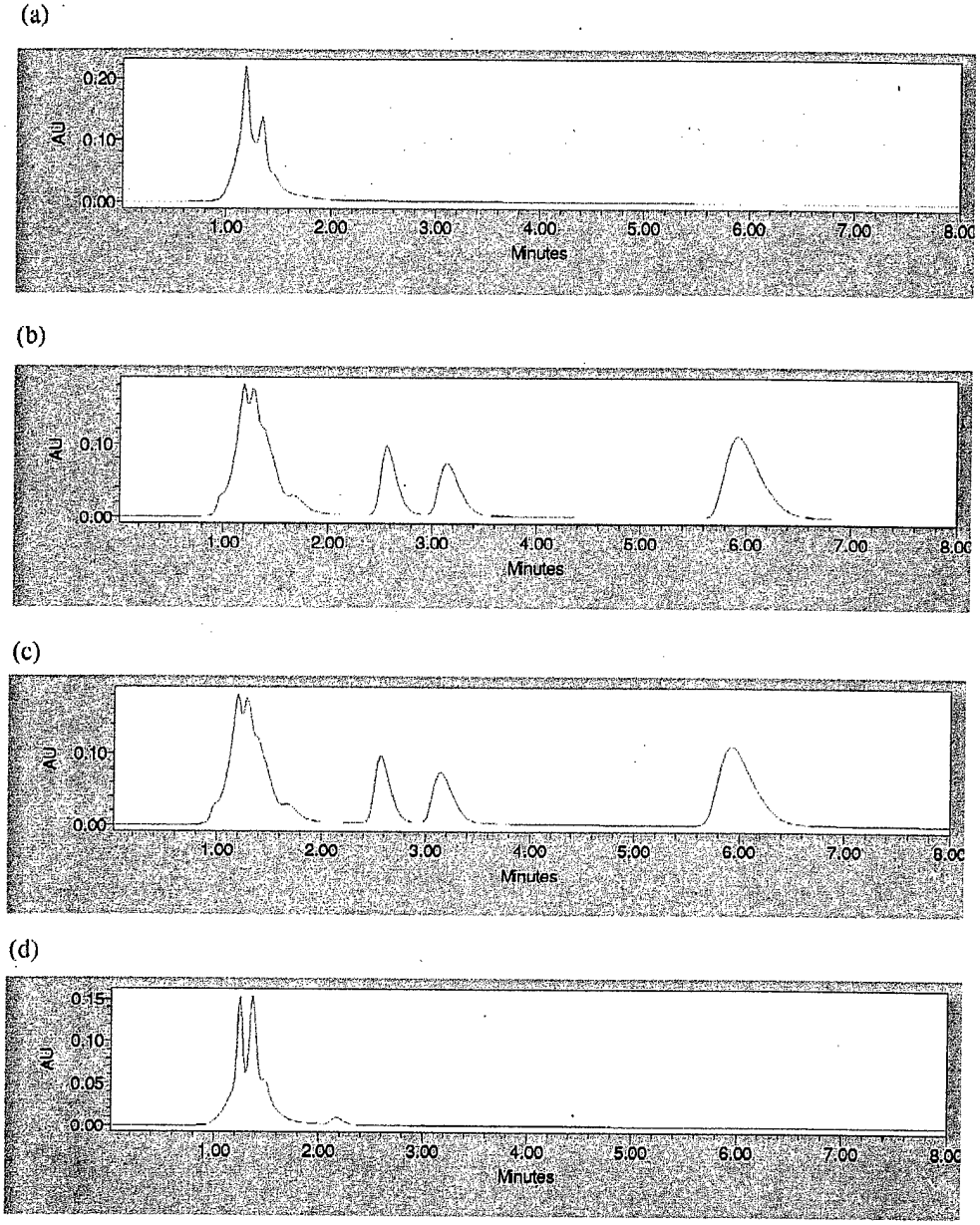



Fig. 3. Typical chromatograms for rat urine samples obtained from the analysis of (a) blank urine. (b) Blank urine with added 1-NITC (10 μg/ml), 1-NA (10 μg/ml), 1-NIC (10 μg/ml), and NE (40 μg/ml), followed by dilution with ACN; (c) blank urine with added 1-NITC (10 μg/ml), 1-NA (10 μg/ml), and NE (40 μg/ml), following dilution with ACN with the supernatant spiked with 1-NIC (10 μg/ml). (d) A urine sample obtained 2–4 h after an i.v. bolus of 25 mg/kg 1-NITC. Chromatographic peaks were identified with the aid of pure reference standards based on retention time $t_R$, including 1-NA (2.2–2.6 min), NE (3.2 min), and 1-NITC (6.0 min).

of α-naphthylisothiocyanate (1-NITC) and two metabolites α-naphthylisothiocyanate (1-NA) and α-naphthylisocyanate (1-NIC) in rat plasma and urine has been developed. The features of the assay include the use of a reversed-phase column, UV detection, protein precipitation using ACN, and the

Table 1
The lower limit of quantitation of 1-NITC, 1-NA, and 1-NIC in rat plasma, urine and ACN

| Compounds | LLQ in plasma (ng/ml) | LLQ in urine (ng/ml) | LLQ in ACN (ng/ml) |
|---|---|---|---|
| 1-NITC | 10 | 30 | 10 |
| 1-NA | 30 | 100 | 30 |
| 1-NIC | ND | ND | 30 |

ND: not detected in blank plasma and urine samples.

use of an internal standard. Through an extensive evaluation of the stabilities of 1-NITC and its metabolites in different biological matrices, we have optimized the conditions for the collection and storage of biological samples.

Based on the features of chemical structures (Fig. 1), naphthylene (NE) was selected as an ideal internal standard candidate. Additionally we found that other chemically unrelated compounds, such as acetophenone and propiophenone, could also be used as the internal standard in this assay. A single UV wavelength of 305 nm was used for the detection of 1-NITC, 1-NA, and 1-NIC in rat plasma and urine samples since we obtained the greatest sensitivity and minimal interference by endogenous compounds present in plasma and urine at this wavelength. Under the current HPLC conditions, the LLQ values were 0.5 ng (10 ng/ml) and 1.5 ng (30 ng/ml) for 1-NITC in plasma and urine, 1.5 ng (30 ng/ml) and 5 ng (100 ng/ml) for 1-NA in plasma and urine, respectively (Table 1).

The extraction of plasma samples was optimized by the use of a protein precipitation step with ACN at 4 °C. Using protein precipitation of plasma samples was more convenient and time-saving than liquid–liquid extraction and solid-phase extraction, and resulted in the least amount of interference with endogenous compounds, while retaining high extraction efficiency. Other organic solvents, such as methanol and acetone, were also investigated in our preliminary studies but produced endogenous interferences and/or variability in recovery. An extraction step for urine samples using ACN, methanol, acetone, and acetyl acetate (EtOAc) was also investigated, since the direct injection of urine supernatant resulted in tailing peaks of 1-NITC, 1-NA, and NE (data not shown). Extraction of urine samples with ACN at 4 °C resulted in the best accuracy, precision, and recovery.

The isothiocyanate group (N=C=S) in 1-NITC and the isocyanate group (N=C=O) in 1-NIC are highly reactive, undergoing hydrolysis. Therefore, the stabilities of 1-NITC, 1-NA, and 1-NIC were systematically investigated with regards to matrix and temperature effects over time. 1-NA was stable in all tested matrices at all tested temperature. However,

Table 2
The within- and between-day accuracy, precision, and recovery for 1-NITC in rat plasma

| | QC (ng/ml) | Accuracy (%) | Precision (%) | Recovery (%) |
|---|---|---|---|---|
| Within-day | 10 | 106 | 97.6 | 93.2 |
| | 500 | 97.5 | 99.8 | 97.4 |
| | 5000 | 95.4 | 98.9 | 94.6 |
| Between-day | 10 | 102 | 92.9 | 95.9 |
| | 500 | 97.3 | 96.5 | 96.7 |
| | 5000 | 99.3 | 96.7 | 96.1 |

Table 3
The within- and between-day accuracy, precision, and recovery for 1-NA in rat urine

| | QC (ng/ml) | Accuracy (%) | Precision (%) | Recovery (%) |
|---|---|---|---|---|
| Within-day | 100 | 106 | 98.7 | 102 |
| | 5000 | 98.2 | 99.4 | 107 |
| | 50 000 | 100 | 98.4 | 110 |
| Between-day | 100 | 105 | 97.4 | 95.4 |
| | 5000 | 96.3 | 97.7 | 104 |
| | 50 000 | 100 | 99.3 | 107 |

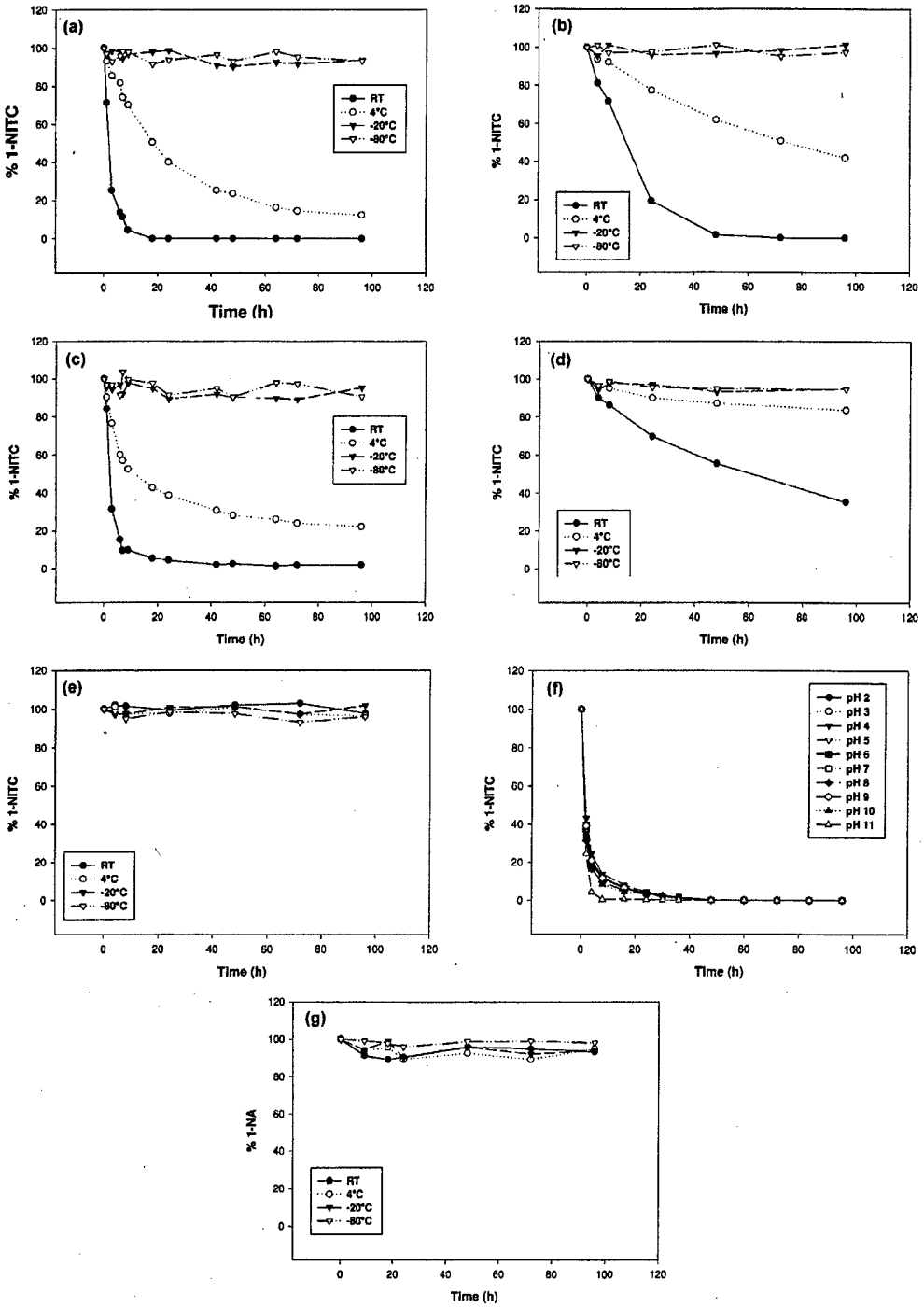



Fig. 4. The stability of 1-NITC, 1-NA, and 1-NIC in rat plasma, urine, ACN precipitated plasma and urine, ACN, and universal buffer at RT, 4, −20, and −80 °C over 96 h. (a) The stability of 1-NITC in rat plasma at RT, 4, −20, and −80 °C. (b) The stability of 1-NITC in ACN precipitated rat plasma at RT, 4, −20, and −80 °C. (c) The stability of 1-NITC in rat urine at RT, 4, −20, and −80 °C. (d) The stability of 1-NITC in ACN diluted rat urine at RT, 4, −20, and −80 °C. (e) The stability of 1-NITC in ACN at RT, 4, −20, and −80 °C. (f) The stability of 1-NITC in universal buffer pH 2–11 at RT. (g) The stability of 1-NA in rat plasma at RT, 4, −20, and −80 °C.

Table 4
Concentrations of 1-NITC in rat plasma samples following a 25 mg/kg i.v. dose

| Time | Conc. (ng/ml) |
|---|---|
| 5 min | 10 490±400 |
| 10 min | 7405±498 |
| 20 min | 4172±140 |
| 30 min | 3312±118 |
| 1 h | 1692±77 |
| 2 h | 1016±48 |
| 4 h | 702±36 |
| 6 h | 620±29 |
| 9 h | 351±15 |
| 12 h | 150±12 |
| 24 h | |

Data is mean±SD; $n=3$.

Table 5
Urinary excretion of 1-NA following a 25 mg/kg i.v. dose of 1-NITC to a female rat

| Time interval (h) | Vol. (ml) | Conc. (μg/ml) | Amount (μg) |
|---|---|---|---|
| 0–2 | 8.2 | 0.42±0.04 | 3.44±0.33 |
| 2–4 | 3.8 | 2.02±0.23 | 7.68±0.87 |
| 4–6 | 1.5 | 2.64±0.35 | 3.96±0.52 |
| 6–9 | 1.5 | 2.17±0.25 | 3.26±0.37 |
| 9–24 | 30 | – | – |
| 24–25 | 3.2 | – | – |
| 0–25 | | | 18.34±2.09 |

Data is mean±SD, $n=3$. –: below detection limit.

the stabilities of 1-NITC and 1-NIC varied under different experimental conditions. The stability of 1-NITC was temperature-dependent in plasma, urine and ACN extracts of plasma and urine, i.e. stable at −20 and −80 °C but degraded at RT and 4 °C. Therefore, the plasma and urine samples obtained in our animal study were centrifuged at 4 °C and stored immediately at −80 °C. The standards of 1-NITC in plasma and urine for calibration curves and QCs were prepared individually on ice and assayed immediately at 4 °C using an autosampler. Under these conditions, the degradation of 1-NITC was less than 5% within 1 h for plasma samples and within 4 h for ACN extracts of plasma at 4 °C.

Our stability studies showed that 1-NITC and 1-NA were stable in plasma and urine at −80 °C when stored for more than 2 months (data not shown). The temperature-independent stability of 1-NITC in ACN indicated that ACN is an ideal extraction solvent for 1-NITC. In addition, the pH-independent degradation of 1-NITC in universal buffer further confirmed its high lability to hydrolysis. The degradation of 1-NITC at pH values of 2–10 was very similar to that of 1-NITC in plasma and urine samples at RT.

The isocyanate group was more reactive than the isothiocyanate group based on our study results. 1-NIC instantly degraded in aqueous matrix, i.e. plasma, urine, ACN precipitated plasma and urine,

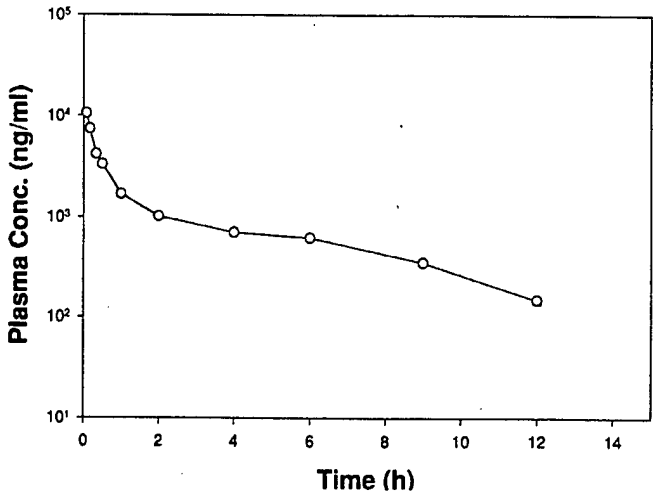



Fig. 5. Log plasma concentration vs. time relationship for 1-NITC after an i.v. bolus dose of 25 mg/kg.

and universal buffer. Although the degradation product of 1-NIC in plasma, urine, and ACN precipitated plasma and urine was not identified, the degradation product in universal buffer was confirmed to be 1-NA. In addition, the information on the stability in ACN indicated that 1-NIC ($t_R$ 3.7 min) is stable in the mobile phase (ACN–$H_2O$ 70:30, v/v) for at least 4 min, but probably shorter than 15 min (10 min for sample preparation and 5 min for mobile phase elution). Therefore the lack of detection of 1-NIC was probably due to its instability in the plasma and urine samples.

Using this HPLC assay, the concentrations of 1-NITC and 1-NA in rat plasma and urine, respectively, were determined (Tables 4 and 5). Our results agree with previous investigations demonstrating no unchanged 1-NITC in urine samples [12]. Analysis of plasma samples allowed the determination of the pharmacokinetic parameters for 1-NITC (clearance of 2.07 l/kg/h, apparent volume of distribution of 14.3 l/kg, and elimination half life of 4.76 h). The metabolite 1-NA was present in urine samples but the total recovery was low (0.4% of the injected dose of 1-NITC) indicating that 1-NITC and its metabolites may be eliminated by other mechanisms such as biliary excretion and $CO_2$ expiration, as reported by Capizzo and Roberts [11]. As well, there may be other unidentified metabolite(s) in urine rather than 1-NA.

## 5. Conclusion

In this paper, we have described a reversed-phase HPLC method for the quantitative determination of 1-NITC and metabolites 1-NA and 1-NIC in rat plasma and urine. The sample pretreatment procedure is based on a rapid precipitation step with ACN for both plasma and urine, thereby eliminating the need of laborious liquid–liquid extraction and solid-phase extraction techniques. The assay provides high sensitivity with LLQ values of 10, 30 and 10 ng/ml for 1-NITC in plasma, urine and ACN. The analysis method is precise and accurate, with the within- and between-day precision and accuracy within the range of 90–110% for QCs at low, medium and high concentration levels. The stability studies showed that 1-NITC was stable at all tested temperatures in ACN, and at −20 and −80 °C in plasma, urine, and ACN extracts of plasma and urine, but degraded at RT and 4 °C. In universal buffer (pH 2–12) at RT, 1-NITC degraded with similar patterns at pH values ranging from 2 to 10; there was rapid degradation at pH 12. 1-NA was stable in all tested matrix at all temperatures (RT to −80 °C). 1-NIC was unstable with rapid degradation in plasma, urine, and ACN extracts of plasma and urine; however, 1-NIC was stable in ACN. The HPLC assay was successfully used in a preliminary rat pharmacokinetic study to analyze plasma and urine samples following the i.v. administration of 25 mg/kg 1-NITC.

## Acknowledgements

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# Efflux Transporters in Drug Excretion

**Shuzhong Zhang and Marilyn E. Morris**

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York, Amherst NY 14260 USA

## Introduction

Therapeutic agents or other xenobiotic compounds will exert their pharmacological or toxicological activities only when sufficient concentrations of these compounds are present at the site of action, where they can bind to the targeted receptors or enzymes. Therefore, the ability of drug molecules to cross biological membranes represents an important determinant of their absorption, distribution, elimination and ultimately their therapeutic or toxic effects. It is clear that the complex biological membrane system is not just pure lipid bilayers, but lipid bilayers embedded with numerous proteins, including transporters. Thus, for a large number of drug molecules, their ability to pass through biological membranes is not solely determined by their physiochemical parameters such as lipophilicity, but also governed by the transporter activities. Among these transporters, a group of so-called efflux transporters (Table 1), including P-glycoprotein, multidrug resistance-associated proteins (MRPs) and breast cancer resistant protein (BCRP) are of particular interest in that they actively remove a wide range of structurally and functionally distinct molecules out of the cells against a concentration gradient. Their transport activities towards a number of clinically important anticancer agents, such as doxorubicin, paclitaxel and vinblastine prevent the intracellular accumulation of these cytotoxic agents and lead to inefficient cell killing, a phenomenon known as multidrug resistance (MDR), which remains the primary obstacle to the successful cancer chemotherapy (*1-4*). In addition, studies characterizing the molecular and functional properties and physiological functions of these transporters, have revealed that these efflux transporters, apart from mediating MDR, play an essential role in governing the absorption, and the intestinal, hepatobiliary and renal excretion of a variety of endogenous and exogenous

compounds (*5-9*). The localization of these efflux transporters in the luminal side of the blood-brain barrier, blood-testis barrier and placenta suggests their central role in regulating the entry of potentially harmful compounds into these pharmacological sanctuaries. It is widely accepted that at least some of these transporters constitute an essential component for the barrier functions between the blood and various tissues and determine the passage of drug molecules or other compounds into these tissues (*5-9*). Furthermore, considering the impact of these transporters on drug disposition, their wide substrate spectrum and their potential saturability, adverse drug interactions due to competive inhibition or induction of these efflux transporters by coadministered drugs, ingested food or environmental compounds could be expected, and this has been proven in a number of in vivo animal or clinical studies (*10-14*). On the other hand, these transport interactions, may also result in beneficial interactions and improve the therapeutic efficacy of a particular drug of interest. For example, the poor bioavailability of some anticancer agents could be improved by inhibiting intestinal P-glycoprotein or other efflux transporters (*15-17*). Lastly, it has been shown that the expression of these efflux transporters varies substantially between individuals, and this variability could be due to the age and gender difference, genetic polymorphism or prior exposure to drugs, food and environmental compounds (*13, 18-22*). The impact of this variability in the expression of these transporters on drug pharmacokinetics remains the topic of extensive investigation and the results obtained from these studies will have significant impact on future therapy. To appreciate the importance of the efflux transporters in drug therapy, an understanding of the molecular and functional characteristics of these transporters and their tissue distribution as well as an appreciation of their impact on drug disposition is essential. This is the focus of the present overview.

## 1. P-glycoprotein

P-glycoprotein is a membrane efflux transporter protein discovered by Juliano and Ling in 1976 from the plasma membrane of Chinese hamster ovary cells selected for resistance to colchicine (*23*). These cells also displayed pleiotropic cross-resistance to a wide range of amphiphilic drugs with distinct

structure and function, a phenomenon nowadays known as multidrug resistance (MDR). The consistent observation of this membrane protein in several MDR cell lines selected with different drugs (*23-26*) and the positive correlation found between the level of P-glycoprotein expression and drug resistance in a variety of MDR cell lines (*27, 28*) strongly suggested that P-glycoprotein may play a key role in mediating MDR. This was subsequently confirmed by gene transfer studies (*29, 30*), in which transfection of P-glycoprotein cDNA was shown sufficient to confer the MDR phenotype upon otherwise drug sensitive cells. The mechanism by which P-glycoprotein mediates MDR is believed to be that P-glycoprotein functions as an ATP-dependent efflux pump, actively extruding a wide range of cytotoxic agents, such as anthracyclines, vinca alkaloids, epipodophyllotoxins and taxol, from inside the cell to the extracellular space, resulting in inadequate intracellular accumulation of these agents for efficient cell killing (*1, 31-34*). It is well established that P-glycoprotein overexpression is one of the major mechanisms responsible for the development of MDR (*2, 35*). The clinical relevance of this MDR mechanism was substantiated by the findings that P-glycoprotein was often detected in numerous resistant human tumors and the expression of this protein represents a poor prognosis factor (*36-44*).

The genes encoding P-glycoprotein have been cloned and belong to a small family of closely related genes designated as mdr. The family consists of two members (MDR1 and MDR3) in humans, and three members (*mdr1a, mdr1b and mdr2*) in rodents (*45-48*). Despite the high homology shared between different members of the family, only human MDR1 and its mouse homologue *mdr1a* and *mdr1b* protein can confer multidrug resistance and drug transport capabilities, while human MDR3 and its mouse homologue *mdr2* protein apparently can not (*29, 30, 47, 49-54*). The latter was shown to be more concentrated in the liver canalicular membranes and functions as a phosphostidylcholine translocase or flippase (*55-58*). Human P-glycoprotein has 1280 amino acids and the polypeptide component of the protein has a molecular weight of 120 to 140 KD (*45*). The apparent molecular weight of P-glycoprotein, however, could vary between 130-190 KD, depending on the level of glycosylation. The molecular structure of the protein was predicted to consist of two homologous halves, each consisting of six transmembrane

domains, and a hydrophilic nucleotide binding domain with Walker A, Walker B and ABC signature sequences, characteristic of ABC proteins (Fig. 1). The nucleotide binding sites are located intracellularly and exhibit ATPase activity, which hydrolyzes ATP and provides the energy for the pumping function of the protein (*59, 60*).

One of the distinctive features of P-glycoprotein from conventional drug transporters is its broad spectrum of substrate specificity (Table 2). These substrates include anticancer agents (e.g., anthracyclines, vinca alkaloids, epipodophylotoxins and taxol) (*2*), cardiac drugs (e.g., digoxin, quinidine) (*61, 62*), HIV protease inhibitors (e.g., saquinavir, indinavir, ritonavir) (*63*), immunosupressants (e.g., cyclosporine) (*64*), antibiotics (e.g., actinomycin D) (*65*) steroids (e.g. cortisol, aldosterone, dexamethasone) (*66, 67*) and cytokines (e.g., IL2, IL-4, IFN-$\gamma$) (*68*). The list of P-glycoprotein substrates could be expanded to include many more compounds. The only common characteristics of these substrates are that most of these compounds are hydrophobic, positively charged or neutral compounds with planar structure (*2, 69*); however, negatively charged compounds, such as methotrexate and phenytoin, can also serve as substrates under certain circumstances (*70-72*). How P-glycoprotein recognizes such a wide range of structurally unrelated chemical entities still remains an enigma, but could be partly owing to the multiple drug binding sites present in the transmembrane domains of the protein (*73-76*). The proposed mechanism by which P-glycoprotein performs its transport function is the so-called "hydrophobic vacuum cleaner" model or the "flippase" model (*2, 77, 78*). In the "hydrophobic vacuum cleaner" model, P-glycoprotein binds directly to its substrates within the plasma membrane and pump them out of the cells (*2*). In the "flippase" model, the binding of substrates takes place in the inner leaflet of the plasma membrane bilayer and the substrates are flipped by P-glycoprotein to the outer leaflet, from which they diffuse into the extracellular space (*77, 78*). In either case, the substrates are removed directly from the cell membrane by P-glycoprotein before their entry into the cytoplasmic solution. The high local concentrations of the hydrophobic compounds in the lipid membrane may facilitate the transport by P-glycoprotein even in the absence of high affinity binding, and this may also help to explain such a diverse substrate spectrum (*79*).

A wide range of P-glycoprotein inhibitors, that are as chemically diverse as the substrates, has also been identified. These inhibitors include calcium channel blockers (e.g., verapamil, diltiazem) (*80*), calmodulin antagonists (e.g., trifluoperazine, fluphenazine) (*81, 82*), steroidal compounds (e.g., progesterone, tamoxifen) (*83, 84*), immunosuppressive agents (e.g., cyclosporin A, FK506) (*85, 86*), antibiotics (e.g., cefoperazone, erythromycin) (*87, 88*) and nonionic detergents (e.g., Triton-X100, Nonidet P-40) (*89*). Interestingly, a number of pharmaceutical excipients such as cremophor EL, Tween 80, and polyethylene glycols were also shown to inhibit P-glycoprotein (*90, 91*). More recently, the list of these inhibitors has been extended to include many dietary compounds in a variety of natural products, such as flavonoids (*92-95*), curcumin (*96*) and piperine (*97*). Many of these inhibitors have undergone clinical testing for their ability to restore tumor responsiveness to chemotherapeutic agents by blocking P-glycoprotein; however, the toxicities associated with the high concentrations of these inhibitors required for a significant P-glycoprotein inhibition have prevented their clinical use. The newly-developed second and third generations of P-glycoprotein inhibitors such as PSC833 (*98*), GF120918 (*99*), LY335979 (*100*) and XR9576 (*101*) have very high potency and low toxicity, and clinical trials using these agents as chemosensitizers have produced some promising results (*102-105*).

The expression of P-glycoprotein is not limited to MDR tumor cells. High levels of expression have been also detected in a number of normal tissues, such as the liver, kidney, gastrointestinal tract, the blood-brain and blood-testis barriers, as well as the adrenal glands (*106-109*). At the subcellular level, P-glycoprotein has been shown to be predominantly located on the apical surface of the epithelial (or endothelial) cells with a specific barrier function, such as the endothelial cells of the blood capillaries in the brain, the canalicular membranes of the hepatocytes, the brush border membranes of renal proximal tubules, and the luminal membrane of the enterocytes in the colon and jejunum (*106, 108, 109*). The polarized expression of this protein in the excretory organs (liver, kidney and intestine) and blood-tissue barriers, together with its ability to transport a wide diversity of chemicals, indicates that the protein may play an important role in protecting the body or certain tissues (such as brain and testis)

from the insult of ingested toxins and toxic metabolites, by actively excreting these toxic agents into bile, urine and intestine, or by restricting their entry into the brain and other pharmacological sanctuaries. P-glycoprotein was also found in placental trophoblasts from the first trimester of pregnancy to full term, indicating it may be also involved in the protection of the developing fetus (*2*).

The role of P-glycoprotein in manipulating excretion and distribution of xenobiotics was initially supported by a number of in vivo animal or clinical studies using a combination of P-glycoprotein substrate drugs and inhibitors, in which a reduced elimination and a increased tissue accumulation of the substrate drugs by the co-administered inhibitors were often observed (*110-112*). However, due to the possible interactions between these inhibitors and other drug transporters or drug metabolizing enzymes and since the inhibitors used in these early studies were relatively non-specific, other interpretations could not be excluded. The most convincing evidence is from a series of elegant studies conducted by Schinkel et. al. (*62, 113, 114*). using knockout mice. Both *mdr1a* (-/-) and *mdr1a/1b* (-/-) knockout mice have been created by disruption of *mdr1a*, or both *mdr1a* and *mdr1b* genes. These knockout mice appeared to be viable, healthy and fertile with normal histological, hematological, and immunological parameters, indicating that mdr1-type P-glycoprotein may not be essential for basic physiology (*113, 114*). However, the mice lacking mdr1 type P-glycoprotein did show hypersensitivity to xenobiotic toxins. For example, the *mdr1a* (-/-) mice were 50-100-fold more sensitive to ivermectin, an acaricide and anthelmintic drug, compared to the wild type mice, and this increased toxicity could be explained by the 90-fold increase in the brain accumulation of ivermectin in the knockout mice, since the toxicity of ivermectin results from its interaction with a neurotransmitter system in the central nervous system (CNS) (*113*). Another interesting example is related to the antidiarrheal drug loperamide, which is a P-glycoprotein substrate. Although loperamide is a typical opioid drug, in humans and animals this drug only demonstrates peripheral opiate-like effects on the gastrointestinal tract with little effect in the CNS due to its inability to pass through the blood brain barrier. After oral administration of loperamide, the *mdr1a* (-/-) mice demonstrated markedly increased CNS opiate-like effects compared with the wild type mice, consistent with a dramatic increase in the brain accumulation of

this drug in the knockout mice (13-fold, $p < 0.001$) (*115*). Interestingly, CNS effects of loperamide in humans were also observed when it was co-administered with quinidine, a competitive inhibitor of P-glycoprotein (*10*). An increased brain accumulation of many other P-glycoprotein substrate drugs such as vinblastine, cyclosporine, digoxin have also been observed in the *mdr1a* (-/-) or *mdr1a/1b* (-/-) mice (*62, 113-115*). Taken together, these data clearly indicate that mdr1-type P-glycoprotein plays a very important role in regulating the entry of xenobiotics or endogenous compounds into the brain. In addition to the marked alterations in the brain accumulation of these P-glycoprotein substrates in the knockout mice, the blood concentrations and the accumulation of these substrates in other tissues such as the liver, heart and intestine were also shown to be significantly elevated, albeit to a lesser extent, indicating a diminished elimination of these compounds in the knockout mice (*62, 113-117*). The high level of P-glycoprotein found in the excretory organs in the body and the diminished elimination of P-glycoprotein substrates observed in P-glycoprotein-deficient mice point to an important role of the protein in the elimination of xenobiotics by these excretory routes.

Xenobiotics can be eliminated from the body by fecal excretion if they are poorly absorbed after oral administration or following secretion into the intestinal lumen. The polarized expression of P-glycoprotein on the apical membrane of the enterocytes lining the intestinal wall (*106*) suggests this efflux transporter is involved in the active secretion of P-glycoprotein substrates into the intestinal lumen and thus facilitates their fecal excretion. In addition, the P-glycoprotein-mediated active efflux of its substrates from the intestinal epithelial cells back to the lumen will also limit the absorption / bioavailability of orally dosed drugs or other compounds that are P-glycoprotein substrates. Significant P-glycoprotein-mediated effects on intestinal secretion and absorption / bioavailability have been observed in a number of studies. In mice, *mdr1a* P-glycoprotein is the major isoform expressed in the intestine and brain (*48, 113*). The plasma AUC of paclitaxel, a known P-glycoprotein substrate, has been shown to be 2- and 6-fold higher in *mdr1a* (-/-) knockout mice than in the wild type mice after i.v. and oral adiministration, respectively. The cumulative intestinal secretion of paclitaxel (0-96 hour) was dramatically decreased from 40% in the wild type animal to < 3% in the knockouts after i.v.

dosing, and the bioavailability of paclitaxel increased from 11% in the wild type mice to 35% in the knockouts after oral dosing (10 mg/kg) (*118*). Similar results have also been obtained for a number of other P-glycoprotein substrates, such as digoxin, grepafloxacin, vinblastine and HIV protease inhibitors (*119-123*). For example, the direct intestinal secretion of $^{3}$H-digoxin was only 2% of the dose in *mdr1a* (-/-) mice, in contrast to 16% in the wild type animals (*119*). Collectively, the results obtained from these knockout animal studies provide convincing evidence for the important contribution of P-glycoprotein to intestinal secretion and absorption of substrate compounds. The clinical relevance of these observations in the animal studies has been demonstrated in several human studies. For example, the intestinal secretion of talinolol, a $\beta_1$-adrenergic receptor blocker, was shown to be against a concentration gradient (5.5 (lumen): 1 (blood)), after its i.v. administration, indicating the involvement of an active process. In addition, the secretion rate of talinolol in the presence of a simultaneous intraluminal perfusion of R-verapamil, a known P-glycoprotein inhibitor, dropped to 29-59% of the values obtained in the absence of R-verapamil (*124*). Similar results have also been obtained for digoxin (*125*). Furthermore, the intestinal secretion of talinolol was also increased significantly in human subjects treated with rifampin, and the increased secretion can be attributed to the 4.2-fold increase in the intestinal P-glycoprotein expression induced by treatment with rifampin (*13*). The oral bioavailability of P-glycoprotein substrates in humans was also shown to be, at least partly, limited by intestinal P-glycoprotein (*20, 126-131*), and co-administration of P-glycoprotein inhibitors or competitive substrates could increase the bioavailability of these substrates (*11, 12*).

Biliary excretion represents another important route for the elimination of drugs and other xenobiotics. Following the uptake of xenobiotics into the hepatocytes, compounds may undergo metabolic modification, or the parent compound, as well as the formed metabolites may be excreted into bile through the canalicular membrane or effluxed back across the sinusoidal membrane into blood. The relatively small surface area of the canalicular membrane (10-15% of the hepatocyte surface area) in contrast to the sinusoidal membrane (at least 70%) and small intracanalicular fluid volume suggests that carrier mediated transport may significantly contribute to the biliary excretion of both

endogenous and exogenous compounds (*7, 132*). Indeed, many active transporters have been identified in the canalicular membrane to mediate this process (*132-134*), including P-glycoprotein and MRP2 (*106, 132*). The contribution of P-glycoprotein to biliary secretion has been demonstrated by several investigations. For example, the biliary excretion of unchanged doxorubicin decreased from 13.3% of the dose in wild type mice to only 2.4% in *mdr1a* (-/-) knockout mice after a 5 mg/kg i.v. dose. (*121*). Similar results have also been obtained for a number of amphiphilic model substrates, which exhibited markedly reduced biliary excretion in both *mdr1a* (-/-) and *mdr1a/1b* (-/-) knockout mice compared to the normal mice (*117, 123*). Studies using P-glycoprotein inhibitors also provided results consistent with the important contribution of P-glycoprotein to biliary excretion. In an isolated perfused rat liver study, erythromycin significantly decreased the biliary excretion of fexofenadine, which is a P-glycoprotein substrate (*135*). Cyclosporin A and its analogue PSC833 have been reported to decrease the biliary excretion of both colchicine and doxorubicin (*136, 137*) in vivo. Similar results have also been observed for doxorubicin and grepafloxacin when the competitive substrates erythromycin (for both doxorubicin and grepafloxacin) and cyclosporin (for grepafloxacin) were administered simultaneously (*138, 139*). In addition, the biliary excretion of P-glycoprotein substrates was shown to depend on the expression level of this protein, and a significant increase in the biliary excretion of vinblastine was observed in rats with increased levels of P-glycoprotein, which was induced by 2-acetylaminofluorene and phenothiazine, respectively, in two independent studies (*140, 141*). These data suggest that P-glycoprotein plays an important role in biliary excretion. However, other studies have failed to find significant effects on P-glycoprotein-mediated biliary excretion in knockout mice. For example, while the intestinal secretion and bioavailability of paclitaxel were markedly altered in *mdr1a* (-/-) knockout mice, the biliary excretion of this model substrate in the knockout mice was not significantly different from that in the wild type animals (*118*). Even in the *mdr1a/1b* (-/-) double knockouts, the biliary excretion of both digoxin and vinblastine was not substantially changed (*114*). One possible explanation of these conflicting results is the presence of alternative transport processes responsible for the secretion of these substrates into bile. P-glycoprotein may act in concert with other transporters in excreting certain substrates into bile,

and the loss of P-glycoprotein function could be compensated for by other transport processes under certain circumstances. Indeed, it has been shown that *mdr1b* expression in the liver and kidney was consistently increased in *mdr1a* (-/-) knockout mice compared to the wild type animals, indicating that the loss of *mdr1a* function could be compensated for by *mdr1b* protein for their common substrates (*113*). Other canalicular membrane transporters may also exhibit overlapping substrate specificity for certain P-glycoprotein substrates.

Renal clearance represents an important route for the elimination of a large number of xenobiotic compounds. This dynamic process includes glomerular filtration, renal tubular secretion and tubular reabsorption. Renal secretion usually takes place against a concentration gradient and thus is mainly an active process involving a variety of transporter mechanisms (*142*). In addition to the two major carrier systems responsible for the renal handling of organic cations and organic anions, several ATP-dependent transporters, including P-glycoprotein and multidrug resistance associated proteins have been detected in the kidney (*142*). The transport function and the localization of P-glycoprotein on the apical membrane of the proximal tubule cells (*106*) suggest the involvement of this protein in the renal secretion of its substrates into urine. The observation that a classic P-glycoprotein inhibitor, cyclosporin, decreased colchicine renal clearance after i.v. administration from 6.23 ± 0.46 to 3.58 ± 0.31 ml/(min•kg) (mean ± SD, $p < 0.05$) without affecting glomerular filtration and the secretion of the organic cation ranitidine or the organic anion p-aminohippurate, provided the first in vivo demonstration for this functional role of P-glycoprotein (*143*). Subsequently, a significant reduction of the renal secretion of digoxin (in rats), vinblastine and vincristine (in dogs) by cyclosporin A was also observed by using the isolated perfused rat kidney or the single pass multiple indicator dilution method (*144, 145*). In humans, the renal clearance of digoxin was decreased by the concomitant use of itraconazole by 20% ($p < 0.01$). Since digoxin is mainly excreted unchanged into urine, this reduction is most likely mediated by the inhibition of P-glycoprotein (*146*). Similarly, the renal clearance of quinidine was also decreased by 50% ($p < 0.001$) by itraconazole in a double-blind, randomized, two-phase crossover study, and inhibition of P-glycoprotein is thought to be the most likely underlying mechanism (*147*). Taken together, these studies

demonstrated that P-glycoprotein significantly contributes to the renal excretion of its substrates.

## 2. Multidrug Resistance-associated Protein (MRP)

The family of human multidrug resistance associated proteins (MRP) is another group of ABC transporters, so far consisting of nine members. Among these members, MRP1, MRP2 and MRP3 have been characterized in some detail in terms of their capability of conferring multidrug resistance and their possible physiological functions (*148*) and so will be the focus of this discussion. The founding member of this family, MRP1, was cloned in 1992 from the resistant human small cell lung cancer cell line (*149*), which does not overexpress P-glycoprotein (*150-153*). Subsequent transfection studies demonstrated that overexpression of this 190 KD membrane protein can confer multidrug resistance against a number of natural product anticancer agents such as the anthracyclines, vinca alkaloids and epipodophyllotoxins, by causing the active efflux of these cytotoxic agents from cells and thus lowering their intracellular concentrations (*154-157*). Later, MRP2 (cMOAT) and other members were also identified and characterized to varying extents (*158-167*). Among these MRPs, MRP3 is the most closely related member to MRP1 with 58% amino acid identity, followed by MRP2 (49%) (*168*). These three MRPs have similar topology, containing a typical ABC core structure of two segments with each consisting of 6 transmembrane domains and an ATP binding domain, similar to P-glycoprotein, and an extra N-terminal segment of five transmembrane domains linked to the core structure through an intracellular loop (*148*) (Fig. 1). Similar to MRP1, both MRP2 and MRP3 have also been shown to be able to confer MDR to several anticancer drugs (*169-172*). The clinical relevance for MRP1-mediated MDR has been a topic of extensive investigation and there is some evidence suggesting that overexpression of MRP1 might represent a poor prognostic factor (*173-181*). The clinical relevance of MRP2- and MRP3-mediated MDR is currently unknown.

In contrast to P-glycoprotein, which mainly transports large, hydrophobic cationic compounds, MRP1 mainly transports amphiphilic anions,

preferentially lipophilic compounds conjugated with glutathione (e.g., leukotrene C4, DNP-SG), glucuronate (e.g., bilirubin, 17β-estradiol), or sulfate (*5*) (Table 2). Some unconjugated amphiphilic anions such as methotrexate and Fluo-3, a penta-anionic fluorescent dye, can also serve as substrates and they are transported in unchanged form (*182, 183*). In addition to the anionic compounds, MRP1 can also accept amphiphilic cations or neutral compounds, such as anthracyclines, etoposide and vinca alkaloids, as its substrates. But paclitaxel, which is a good P-glycoprotein substrate, appears not to be transported (*154-157*). These cationic or neutral substrates are thought to be transported intact but need reduced glutathione (GSH) as a cotransporting factor (*184-187*). As such, depletion of intracellular GSH by buthionine sulphoximine (BSO), an inhibitor of glutathione (GSH) synthesis, can increase the intracellular accumulation of these substrates in MRP1 overexpressing cells (*188, 189*). Both MRP2 and MRP3 share a similar substrate spectrum with MRP1. They can also transport conjugates of lipophilic substances with glutathione, glucuronate and sulfate such as glutathione S-conjugate leukotriene C4, glucuronosyl bilirubin and anticancer agents methotrexate, vincristine and etoposide (*5*). In transporting cationic substrates, MRP2 and MRP3 seem to function by the same mechanism as MRP1 and need GSH as a cosubstrate (*187*). However, the substrate specificity of these three MRP isoforms is not identical, and for their common substrates, the transporting efficiency by these isoforms varies substantially (*5*); there are substrates that can be recognized by one isoform but not the others. For example, cisplatin has been shown to be a substrate for MRP2 but not for MRP1 (*154, 190, 191*); the conjugated monoanionic bile acids glycocholate and taurocholate are substrates for MRP3 but not for MRP1 and MRP2 (*192-194*). In contrast to P-glycoprotein, for which many inhibitors have been identified, there are only a few compounds known to inhibit MRP to a significant degree. The well-known potent P-glycoprotein inhibitors such as GF120918 and LY335979 have little effect on MRP while verapamil, cyclosporin A and PSC833 have been shown to be, at best, moderate MRP inhibitors (*5, 195, 196*). The best-known MRP inhibitor so far appears to be MK571, which is a leukotriene D4 receptor antagonist. MK571 inhibits both MRP1 and MRP2, but a mild stimulatory effect on MRP3-mediated transport of 17β-estradiol glucuronide has been reported (*159, 197, 198*).

To understand the physiological function of these energy-dependent MRP efflux transporters, their normal tissue distribution has been extensively investigated. While MRP1 appears to be distributed in a wide range of tissues throughout the body, MRP2 and MRP3 have been detected mainly in the gut, liver and kidney (*6, 148*). At the subcellular level, MRP1 is predominantly located in the cell plasma membrane, and in polarized epithelial cells such as hepatocytes, enterocytes and endothelial cells, its distribution is confined to the basolateral membranes (*6, 148, 199*). The active transport function of MRP1 towards a number of exogenous and endogenous toxic substrates and its ubiquitous tissue distribution indicate that MRP1 may represent a detoxifying mechanism, protecting some tissues or organs from exposure to toxic substances (*168*). Recent studies using *mrp1* (-/-) knockout mice have provided convincing evidence for this important function. It has been shown that mice with disrupted Mrp1 (*Mrp1* (-/-)) are viable, fertile and have no physiological or histological abnormalities, indicating that Mrp1 may be not essential for normal mouse physiology. However, these *mrp1* (-/-) mice did show a two-fold higher sensitivity to a cytotoxic agent, etoposide, with increased bone marrow toxicity (*200, 201*). A similar observation has also been made by Johnson et. al. (*202*), who demonstrated that a therapeutic dose of vincristine, which normally does not express bone marrow toxicity and gastrointestinal damage, caused extensive damage to these tissues in both *mrp1 (-/-)* and *mdr1a/1b* (-/-) knockout mice, indicating that Mrp1, mdr1-type P-glycoprotein and probably other related efflux transporters work in concert as a detoxifying mechanism to protect tissue from damage induced by toxic agents. In addition, the polarized localization of MRP1 in the basolateral membrane of the choroid plexus epithelium (*203*) suggests that it may significantly contribute to the blood-CSF (cerebrospinal fluid) barrier function, preventing the entry of amphiphilic anions or anticancer drug substrates into CSF. This has also been convincingly demonstrated in a knockout mice study conducted by Wijnholds et. al. (*204*), in which the investigators found that after an i.v. dose of etoposide, the CSF concentration was about 10-fold higher in *mdr1a/mdr1b/mrp1* (-/-/-) triple knockout mice than in *mdr1a/mdr1b* (-/-) double knockout mice, indicating the important contribution of Mrp1 to the blood-CSF barrier function in mice. Taken together, there is strong evidence indicating that MRP1 plays an important role in

protecting the tissue from the damage induced by both exogenous and endogenous toxic substances, and contributes significantly to maintaining the blood-CSF barrier function.

Unlike MRP1, the distribution of MRP2 and MRP3 are restricted to certain tissues such as liver, intestine and kidney (*6, 148*). Similar to P-glycoprotein, MRP2 is exclusively localized to the apical membrane of the polarized cells such as hepatocytes, intestinal epithelial cells and renal proximal tubule cells (*5, 159, 205*), suggesting it may also play a similar role in the secretion of xenobiotics and endobiotics by these excretory routes. The loss of MRP2 in humans is associated with Dubin-Johnson syndrome, a benign hereditary disorder characterized by mild conjugated hyperbilirubinemia and pigment disposition in the liver due to impairment in the MRP2-mediated transport function (*206-209*). Two naturally occurring mutants GY/TR$^-$ and EHBR rats from the Wistar and Sprague-Dawley rat colonies, respectively, also lack Mrp2 expression and are considered animal models for the human Dubin-Johnson syndrome (*158, 160, 210, 211*). Many functional-characterization and substrate-identification studies for MRP2 have been performed by using these Mrp2 deficient rats. It has been shown that the AUC (0-6 hour) of $^{14}$C-temocapril was dramatically increased and the biliary clearance, as measured by total radioactivity, was markedly decreased (0.25 ml/min/kg vs. 5.00 ml/min/kg) in EHBR rats compared with the control Sprague Dawley rats after i.v. administration. Since the active metabolite temocaprilat accounted for > 95% of the total radioactivity, these data indicate that Mrp2 plays a central role in the biliary excretion of the metabolites of this drug (*212*). The biliary excretion of grepafloxacin was also markedly decreased for both parent compound (0.52 vs. 1.79 ml/min/kg) and glucuronide metabolites (0.09 vs. 15.53 ml/min/kg) in EHBR rats compared with the Sprague Dawley rats (*213*). Recently, Chen et al. (*214*) reported that the biliary excretion of methotrexate and probenecid was decreased 39- and 37-fold, respectively, in EHBR rats as compared to control rats. Similar results were also observed for several other drugs or metabolites such as cefodizime, acetaminophen glucuronide, acetaminophen glutathione conjugate and acetaminophen mercapturate, pravastatin, and indomethacin glucuronide (*215-219*). Interestingly, it was shown that the biliary excretion of CPT11, the active metabolite SN-38, and its glucuronide conjugate can be

substantially decreased by probenecid, an MRP2 inhibitor, with concomitant elevation of plasma concentrations of these compounds in normal rats, resulting in decreased GI toxicity (*220*). Collectively, these data strongly suggest the essential role of MRP2 in the biliary excretion of xenobiotics or their metabolites that are MRP2 substrates. The polarized localization of MRP2 in intestinal epithelial cells also suggests its potential contribution to intestinal secretion and to limiting the intestinal absorption of its substrates, leading to a decreased bioavailability. This hypothesis has been supported by the results of a number of studies. For example, after i.v. administration of CDNB (1-Chloro-2,4-dinitrobenzene), the intestinal secretion of DNP-SG (2,4-dinitrophenyl-*S*-glutathione) was negligible in EHBR rats, whereas a small amount of secretion was observed in Sprague Dawley rats, indicating the involvement of MRP2 in the active secretion of DNP-SG into intestinal lumen. This was also confirmed by Ussing chamber studies, in which the serosal-to-mucosal flux of DNP-SG was shown to be 1.5-fold higher than the mucosal-to-serosal flux in Sprague Dawley rats, and no difference in the flux in both directions was observed in EHBR rats (*221*). The decreased intestinal secretion of grepafloxacin in EHBR rats was observed in a study by Naruhashi et. al. (*222*), which was also confirmed by the 2-fold higher flux in the serosal-to-mucosal direction compared with that in the mucosal-to-serosal direction in the Sprague Dawley rats and no differences in the EHBR rats. By analogy with P-glycoprotein, the impact of MRP2-mediated efflux of its substrates from the enterocytes into the lumen can be illustrated by the 2-fold higher absorption of PhIP (p-2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine), a food-derived carcinogen, in Mrp2-deficient rats compared with the normal rats, and the increased bioavailability of PhIP in normal rats treated with BSO, which is a inhibitor for GSH synthesis (*223, 224*). All these studies provide convincing evidence for the important contribution of MRP2 to the intestinal secretion and absorption of drugs. Whether MRP2 is also present in the brain capillary endothelial cells may still need further investigation, but current evidence suggests it most likely is (*225*). Mouse Mrp2 was detected on the luminal surface of the brain capillary endothelium (*226*) and shown to actively transport sulforhodamine 101 and fluorescein methotrexate into the luminal compartment of isolated brain capillary (*226*). This transport process can be inhibited by leukotriene $C_4$, 1-chloro 2,4-dinitrobenzene (a precursor of DNP-SG) and vanadate (an ATPase

inhibitor), but not by P-glycoprotein inhibitors such as PSC833 and verapamil. Therefore, the evidence suggests that human MRP2 may also contribute to the blood-brain barrier function in a similar manner as P-glycoprotein does. MRP3 has a similar tissue distribution as MRP2, but is located on the basolateral surface of the polarized cells (*171, 199, 227*).

The expression of MRP3 in the basolateral membrane of intestinal epithelial cells, hepatocytes and renal proximal tubule cells suggests that it tends to remove the substrates from the cytosol into blood. The impact of this process on drug disposition still remains to be clarified, especially considering its limited expression in the excretory organs under normal physiological condition. Interestingly, it has been shown that MRP3 is significantly up-regulated in the liver of MRP2-deficient rats and in the patients with Dubin-Johnson syndrome or patients with primary bliliary cirrhosis (*227, 228*), indicating MRP3 may serve as a compensatory mechanism to remove the conjugates out of the hepatocytes through sinusoidal membrane under the condition where the MRP2-mediated biliary excretion is impaired (*5*)

**3. Breast Cancer Resistance Protein (BCRP)**

BCRP is a new member of the ABC transporter superfamily initially cloned from a doxorubicin-resistant breast cancer cell line (MCF-7/AdrVp) selected with a combination of adriamycin and verapamil (*229*). Two other groups also independently identified this transporter from human placenta (*230*) and human colon carcinoma cells (S1-M1-80) (*231*), and named the protein ABCP (ABC transporter in placenta) and MXR (mitoxantrone resistance-associated protein), respectively. Molecular characterization revealed that BCRP consists of 655 amino acids with a molecular weight of 72.1 KD. In contrast to P-glycoprotein and MRP1 or MRP2, which contain a typical core structure of twelve transmembrane domains and two ATP binding sites, BCRP only has six transmembrane domains and one ATP binding site (Fig. 1), and therefore appears to be a half ABC transporter (*230*). BCRP is the second member of the ABCG subfamily containing members such as *drosophila white, brown and scarlet genes*, and thus "ABCG2" was recommended by the Human Genome Nomenclature Committee (HUGO) to refer to this newly identified transporter

(*3*). As a half transporter, BCRP most likely forms a homodimer to transport its substrates out of the cells utilizing the energy derived from ATP hydrolysis (*3, 229, 232-234*). The murine homologue of BCRP, Bcrp1, has also been cloned and shown to be highly identical (81%) to BCRP with a virtually superimposable hydrophobicity profile (*235*). In addition, another gene closely related to Bcrp1 has also been identified in mice and named Bcrp2, which shares 54% identity with Bcrp1 (*236*). Whether Bcrp1 forms a heterodimer with Bcrp2 to perform its transport function remains unknown; however, the different expression patterns of these two genes indicate that Bcrp2 is not a necessary component for the transport function of Bcrp1. Distinct from other half transporters such as TAP1 and TAP2 (the transporters associated with antigen presentation), which are localized in the intracellular membranes (*237*), both human BCRP and murine Bcrp1 were shown to be predominantly present in the plasma membrane (*16, 238, 239*). Similar to P-glycoprotein and MRP1, both BCRP and Bcrp1 can be overexpressed in vitro upon drug selection or by transfection of cDNAs encoding these proteins, and confer multidrug resistance by the energy-dependent efflux of its substrates out of cells (*229, 232, 233, 235, 240, 241*). Significant and variable expressions of BCRP have been detected in human tumors such as acute leukemia and breast cancer; however, the contribution of this efflux transporter to the clinical MDR needs to be further investigated (*242-248*).

There is considerable overlap in the substrate specificity among P-glycoprotein, MRP1 or MRP2 and BCRP, although the binding affinity of a particular substrate to these transporters may vary substantially (*3*). The BCRP/Bcrp1 substrates identified so far include a number of anticancer agents such as anthracyclines (e.g., doxorubicin, daunorubicin, epirubicin), epipoxophyllotoxins (e.g., etoposide, teniposide), camptothecins or their active metabolites (e.g., topotecan, SN-38, 9-aminocamptothecin, CPT11), mitoxantrone, bisantrene, methotrexate, flavopiridol and HIV-1 nucleoside reverse transcriptase inhibitors (e.g., zidovudine, lamivudine); vincristine, paclitaxel and cisplatin appear not to be substrates (*8, 229, 235, 249-253*). The amino acid at the 482 position seems to be critical in defining substrate specificity because mutated forms of BCRP with arginine at the 482 position changed to threonine or glycine have shown different substrate preference

(*251*). Whether these mutated forms of BCRP also occur in vivo, especially in normal physiological situations, is currently unknown. However, similar phenomenon observed in mouse cell lines selected with doxorubicin, indicates that the 482 position appears to be a hot mutation spot and thus similar mutations might also happen in human tumors upon drug treatment (*254*). For investigating the pharmacological and physiological functions of BCRP and for MDR reversal, there is substantial effort in searching for and developing potent BCRP/Bcrp1 inhibitors. Fumitremorgin C (FTC) derived from *Aspergillus fumigatus* cultures appears to be the first identified potent and specific inhibitor for BCRP/Bcrp1 (*255, 256*); however, its in vivo application is limited by its neurotoxicity. The typical P-glycoprotein inhibitors, GF120918 and reserpine were also shown to be potent BCRP inhibitors (*235, 257*), but many of the other P-glycoprotein inhibitors such as LY335979, cyclosporin A, PSC833 and verapamil have little effect (*8, 258*). So far, the most potent specific BCRP inhibitor appears to be Ko134, an analogue of FTC (*259*). The compound has been used in vivo and demonstrated little or low toxicity in mice at high oral or i.p. doses, and could potentially be used in vivo for BCRP inhibition (*259*).

Interestingly, the distribution of BCRP in normal tissues is similar to P-glycoprotein. High levels of BCRP expression were detected in the human placenta syncytiotrophoblast plasma membrane, facing the maternal bloodstream, in the canalicular membrane of the liver hepatocytes, the apical membrane of the epithelium in the small and large intestine, in the ducts and lobules of the breast and in the luminal surface of brain capillaries (*260, 261*). In addition, significant amounts of BCRP were also found in venous, capillary, but not arterial, endothelial cells in almost all the tissues investigated (*261*). By analogy with P-glycoprotein, it is reasonable to speculate that one, if not the major physiological function of BCRP is to protect body or certain tissues from the exposure of toxic endogenous or exogenous compounds. The localization of BCRP in the placenta, brain and testis may regulate the entry of its substrates into the developing fetus, brain and other pharmacological sanctuaries, and therefore represents an important component of the blood-placenta, blood-brain and blood-testis barriers. The expression of BCRP in the luminal side of the intestinal epithelial cells and canalicular membrane of the hepatocytes suggests that the protein may play a significant role in intestinal secretion or back efflux

to the intestinal lumen and in biliary excretion, thus limiting the entry of xenobiotic toxins into the systemic circulation or facilitating their elimination. A recent study conducted by Jonker et. al. (*262*), using Bcrp1 (-/-) knockout mice, strongly supports this speculation. In the study, the authors demonstrated that the oral bioavailability of topotecan increased about 6-fold in Bcrp1 (-/-) mice compared with the wild type mice and the accumulation of topotecan in Bcrp1 (-/-) fetuses was elevated 2-fold higher compared with the accumulation in the wild type fetuses (following normalization by the maternal plasma concentration), indicating Bcrp1 plays a critical role in the protection of the fetus from exposure to harmful substances. In addition, the authors also elegantly demonstrated that without functional Bcrp1, mice become at least 100-fold more sensitive to pheophorbide a, a dietary chlorophyll-breakdown product and Bcrp1 substrate, resulting in phototoxicity. The hypersensitivity could be explained by the markedly elevated plasma concentrations of pheophorbide a in these knockout mice, and therefore illustrates the importance of this transporter in the protection against natural toxins. Furthermore, studies from the same group also demonstrated that co-administration of topotecan with GF120918, a Bcrp1 inhibitor, dramatically increased the AUC of topotecan more than 6-fold due to the increased uptake from the intestine and the decreased biliary excretion in the *mdr1a* (-/-) mice (i.e., in the absence of P-glycoprotein) (Fig. 2) (*16*). Similar results have also been obtained when topotecan was co-administered with Ko134 (*259*). In humans, it has been shown that the apparent oral bioavailability of topotecan was significantly increased from 40.0% to 97.1% following the co-administration of GF120918. This change most likely resulted from the inhibition of BCRP; it is known that topotecan is only a weak substrate of P-glycoprotein (*15*). Taken together, there is convincing evidence that BCRP plays an important role in governing the body disposition of xenobiotics. It also should be noted that the expression of BCRP and MRP2 in the human intestine is even higher than P-glycoprotein (*263*), and therefore, it may be possible that the contribution of BCRP to the intestinal secretion and oral absorption of xenobiotics can be comparable with, if not greater than that of P-glycoprotein.

**4. Other efflux transporters (MDR3, BSEP)**

MDR3 is the other human P-glycoprotein isoform with virtually identical molecular structure to that of the human MDR1 and mouse *mdr1b* genes (*46*). MDR3 is mainly present in the canalicular membrane of liver hepatocytes and functions as an ATP-dependent phosphotidylcholine translocator (*55-58*). Initially, it was thought that MDR3 protein and its mouse homologue *mdr2* can not transport drugs and confer multidrug resistance (*53, 54*). But more recently, it has been shown that MDR3 is also capable of transporting several cytotoxic drugs such as digoxin, paclitaxel, and vinblastine, but with a low efficiency (*264*). A defect in MDR3 is believed to be associated with an autosomal recessive hereditary disorder, progressive familial intrahepatic cholestasis type 3 (PFIC3) (*265, 266*). BSEP (SPGP, ABCB11) is another homologue of MDR P-glycoproteins, initially identified from the pig and named the Sister of P-glycoprotein (spgp) (*267*). Subsequently, rat Bsep gene was also cloned and shown to be an ATP-dependent bile salt exporter with a Km value of about 5 μM for transporting taurocholate (*268*). Bsep is almost exclusively present in the liver and localized to the canalicular microvilli and subcanalicular vesicles of the hepatocytes and functions as a major bile salt export pump in mammalian livers (*267*). The functional characterization of human BSEP has also been carried out recently and reported to have a similar $K_m$ value for taurocholate (*269, 270*). A defect in BSEP in humans was associated with type 2 PFIC (PFIC2) (*271-273*). At this time, it is generally believed that both MDR3 and BSEP may not play a significant role in terms of drug disposition.

**Conclusions**

The molecular and functional characterization of efflux transporters, during the last 15 years, has facilitated our understanding concerning how these transporters control the passage of a diverse range of substrates through biological membranes. The characterization of their tissue localization and their function has suggested a significant impact of these transporters on the absorption, elimination and distribution of xenobiotic compounds as a body defense mechanism against the exposure of both endogenous and exogenous toxins. The generation of knockout mice lacking specific transporter(s) and the identification of specific inhibitors has greatly enhanced our ability to understand the physiological and pharmacological functions of these

transporters. It has been clearly demonstrated by the studies presented here, as well as others, that these efflux transporters play an essential role in intestinal absorption, biliary excretion, and renal secretion and contribute to the barrier functions between the blood and various tissues such as brain, testis and placenta. Considering the important impact of these efflux transporters in drug disposition, identification of substrates and inhibitors from commonly prescribed drugs or food-derived compounds and characterization of their kinetic parameters will help to predict potential drug interactions mediated by these transport mechanisms. However, the full appreciation of the impact of these transporters on drug disposition will depend on our understanding of the mechanism(s) by which these transporters recognize such a wide range of structurally distinct substances, the mechanism(s) by which these transporters are regulated, the influence of multiple co-existing transporters, as well as the interplay of these transporters with drug metabolizing enzymes; these aspects are all largely unknown at this time and remain to be investigated.

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Table 1. Characteristics of efflux transporters

| Member | HUGO symbol | Alternative name | Tissue localization | Subcellular level | Associated disease |
|---|---|---|---|---|---|
| MDR1* | ABCB1 | PGY1, P-gp | liver, gut, kidney, adrenal gland, blood brain barrier, placenta | apical | drug resistance |
| MDR3* | ABCB4 | PGY3, MDR2/MDR3 | liver canalicular membrane | apical | PFIC3 |
| MRP1* | ABCC1 | MRP, GS-X | ubiquitous | basolateral | drug resistance ? |
| MRP2* | ABCC2 | cMOAT | liver, intestine, kidney | apical | Dubin-Johnson syndrome |
| MRP3* | ABCC3 | cMOAT2, MLP2, MOAT-D | liver, intestine, kidney, adrenal gland | basolateral | ? |
| BCRP* | ABCG2 | ABCP, MXR | placenta, liver, intestine apical membrane | apical | ? |
| BSEP** | ABCB11 | SPGP | liver canalicular membrane | apical | PFIC2 |

* Data from Litman et. al. *(3)*.
** Data from Trauner and Boyer *(274)*.

Table 2. Common substrates and inhibitors for the efflux transporters

| Transporter | Substrates | Inhibitors |
|---|---|---|
| MDR1 | anthracyclines, vinca alkaloids, epipodophyllotoxins, paclitaxel, topotecan*, mitoxantrone*, HIV protease inhibitors, digoxin, Rhodamine123, methotrexate | verapamil, diltiazem, trifuoperazine, quinidine, reserpine, cyclosporin A, valinomycin, terfenidine, PSC833, VX710*, PAK-104P, GF120918, LY35979*, XR9576* |
| MDR3 | phospotidylcholine, digoxin (?), paclitaxel (?), vinblastine (?) | verapamil+, cyclosporin A+, and PSC833+ |
| MRP1 | Aflatoxin B1, doxorubicin, etoposide, vincristine, methotrexate, and various lipophilic glutathione, glucuronide and sulfate conjugates | MK571, cyclosporin A, VX710*, PA-104P* |
| MRP2 | glutathione conjugates, glucuronides, sulfate conjugates, methotrexate, temocaprilat, CPT11 arboxylate, SN38 carboxylate, cisplatin, pravastatin | MK571, cyclosporin A |
| MRP3 | glutathione conjugates, glucuronides, sulfate conjugates, methotrexate, monoanionic bile acids (taurocholate, glycocholate), vincristine, etoposide | MK571 (?) |
| BCRP | anthracyclines, epipoxophyllotoxins, camptothecins or their active metabolites, mitoxantrone, bisantrene, methotrexate, flavopiridol, zidovudine, lamivudine | FTC, GF120918, Ko-134 |
| BSEP | bile salts | |

Data were taken * *(3)* or + *(263)*.

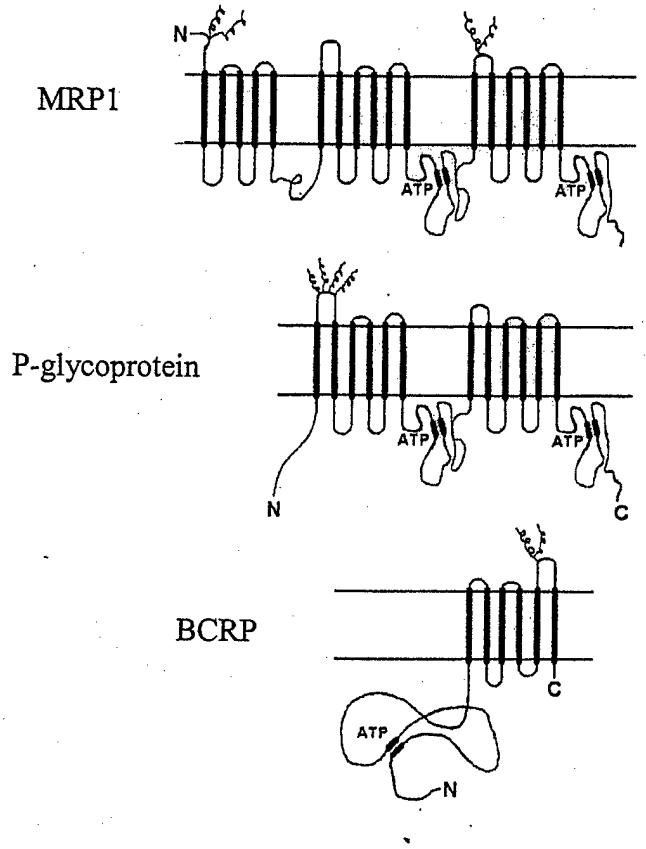


Fig. 1. Topology of efflux transporters MRP1, P-glycoprotein and BCRP (Reproduced with permission from ref *3*)

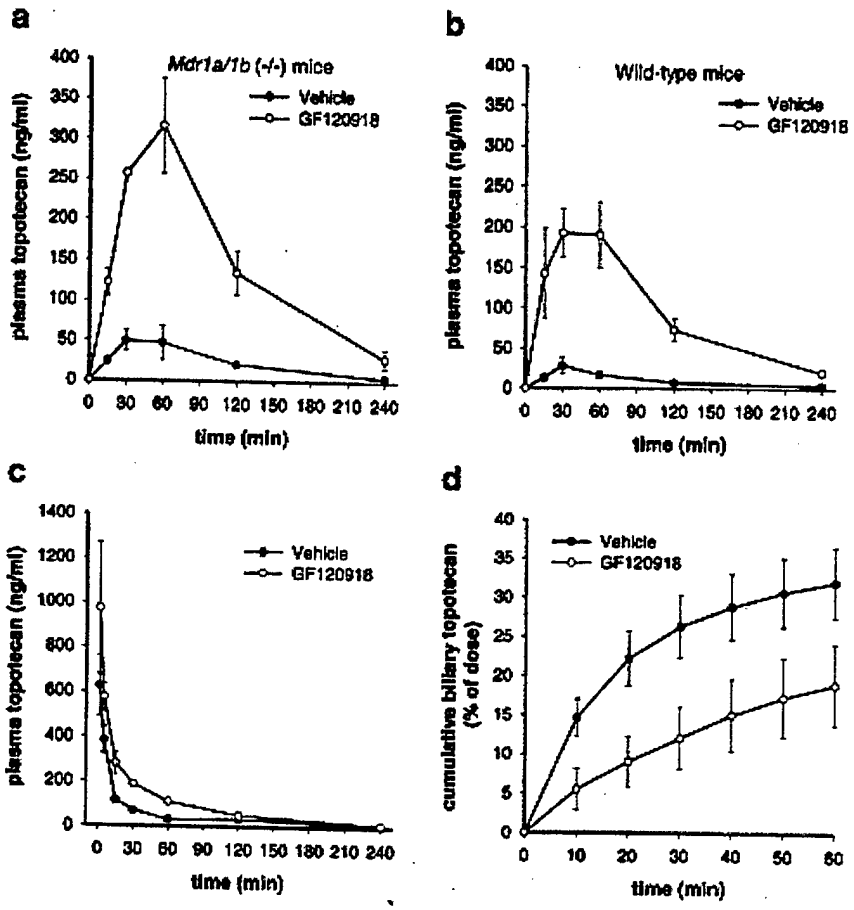


Fig. 2 Effects of GF120918 on the plasma concentration and biliary excretion of topotecan in mice

*Mdr1a/1b* (-/-) (**a**) or wild-type (**b**) mice were given an oral dose of GF120918 (50 mg/kg) or vehicle 15 minutes before an oral dose of topotecan (1 mg/kg). (**c**) *Mdr1a/1b* (-/-) mice were given an i.v. dose of topotecan in combination of an oral GF120918 or vehicle. (**d**) Cumulative biliary excretion of topotecan in *mdr1a/1b* (-/-) mice treated in the same way as (**c**). Results are the means ± SD (n ≥3). (Reproduced with permission from ref *16*).

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# Determination of Phenethyl Isothiocyanate in Human Plasma and Urine by Ammonia Derivatization and Liquid Chromatography-Tandem Mass Spectrometry

Yan Ji and Marilyn E. Morris*

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York, Amherst, New York, 14260

Correspondence and Reprints:

Marilyn E. Morris, Ph.D.
Department of Pharmaceutical Sciences
517 Hochstetter Hall
University at Buffalo
State University of New York
Amherst NY, 14260-1200
Tel: (716)645-2842 ext 230
Fax: (716)645-3693
e-mail: memorris@buffalo.edu

Running Title: LC/MS/MS Assay of Phenethyl Isothiocyanate

Category: Chromatographic Techniques

**Abstract**

Phenethyl isothiocyanate (PEITC) is a dietary compound present in cruciferous vegetables that has cancer preventive properties. Our objective was to develop and validate a novel liquid chromatography-tandem mass spectrometry (LC/MS/MS) procedure to analyze PEITC concentrations in human plasma and urine. Following hexane extraction, ammonia was added to samples to derivatize PEITC to phenethylthiourea. Chromatographic separation was achieved on a $C_{18}$ column with acetonitrile/5 mM formic acid (60:40, v/v) as the mobile phase followed by tandem mass spectrometry detection in multiple reaction monitoring mode. Deuterium-labeled PEITC was used as the internal standard. The detection limit was 2 nM and calibration curves were linear from 7.8 to 2000 nM. The intra- and inter-day coefficients of variation were less than 5% and 10% respectively. The intra- and inter-day accuracy ranged from 101.0 to 104.2% and 102.8 to 118.6%, respectively. The recovery from spiked human plasma and urine ranged from 100.3 to 113.5% and 98.3 to 103.9%, respectively. The assay was used to measure PEITC in plasma and urine samples obtained from subjects after consumption of 100 g of watercress. This novel assay represents the first analytical method with the sensitivity and specificity to determine plasma and urine concentration of PEITC.

Key Words: PEITC; phenethyl isothiocyanate; LC/MS/MS; pharmacokinetics; plasma; urine.

Organic isothiocyanates (ITCs)[1] are chemopreventive compounds occurring in a wide variety of cruciferous vegetables as glucosinolates. Damage to plant cells, such as from cutting and chewing, releases myrosinase that catalyzes the hydrolysis of glucosinolates and the formation of ITCs by a Lossen rearrangement [1]. Phenethyl isothiocyanate (PEITC) (Fig. 1) is one of the most extensively studied ITCs because of its high potency against a variety of tumors and its low *in vivo* toxicity. Human exposure to PEITC is primarily through consumption of certain cruciferous vegetables containing its glucosinolate precursor, gluconasturtiin. Numerous cell and animal studies have demonstrated effective chemoprevention activity of PEITC for cancers, such as those of the lung, breast, esophagus, forestomach, pancreas, prostate, colon, and with leukemia [2-4]. In addition, no toxicity has been observed in animal models with equivalent anticarcinogenic doses or at higher doses [1]. Based on promising animal studies regarding the efficacy and toxicity of PEITC, the compound is being studied in a Phase I clinical trial conducted by National Cancer Institute, for its ability to prevent lung cancer in smokers and ex-smokers [5]. However, no study has specifically measured the concentration of unchanged PEITC in clinical samples. This information is necessary in order to evaluate *in vivo* concentration-effect and concentration-toxicity relationships and to design dosing regimens. Therefore, there is a need for an analytical method with the specificity and sensitivity to quantitate PEITC in human plasma samples.

Existing methods to analyze PEITC include high-performance liquid chromatography (HPLC), gas chromatography (GC) [6], gas chromatography-mass spectrometry (GC/MS) [7], and HPLC utilizing cyclocondensation derivatization [8-11]. When 40 mg of oral PEITC was administrated to humans, total ITC plasma concentrations were mainly within the nanomolar range [10]. HPLC and GC methods are not sensitive enough to analyze clinical samples containing this

concentration of PEITC. GC/MS is able to measure individual ITCs [7]; however, the sensitivity of GC/MS is inadequate due to instrumental limitations as well as the volatility of PEITC that causes loss of the analyte during sample extraction. The HPLC-based cyclocondensation approach enhanced sensitivity significantly by derivatizing ITCs to 1,3-benzenedithiol-2-thione through a cyclocondensation reaction (Fig. 2A) and has been applied to analyzing plant materials, urine, and plasma. However, the assay lacks specificity for analyzing a particular ITC, since any ITC or dithiocarbamate (DTC) will form the identical product to be detected (Fig. 2A). Vegetables usually contain more than one ITC. Hence, if a vegetable such as watercress or broccoli sprouts is given to human subjects, the plasma profile of a specific ITC of interest (*i.e.*, PEITC or sulforaphane) cannot be obtained. Even if a specific type of ITC is administered to subjects and food restriction is carried out before initiation of the study, there may still be trace amounts of other ITCs and DTCs present in the plasma due to the extensive exposure of humans to DTCs as fungicides, insecticides, pesticides and rubber vulcanization accelerators [11] as well as the complexity of human diets. Additionally, the major metabolite of PEITC, PEITC-N-acetylcysteine (PEITC-NAC) (Fig. 1), is also a DTC compound [12]. Therefore, when used to analyze plasma or urine concentrations of PEITC, the cyclocondensation approach would not be able to distinguish PEITC from its metabolites including PEITC-NAC, other ITCs or dithiocarbamates (DTCs).

In this study, we developed and validated a novel analytical approach that involves ammonia derivatization of PEITC to phenethylthiourea (Fig. 2B) and liquid chromatography-tandem mass spectrometry (LC/MS/MS) quantitation. The method is able to analyze PEITC in human plasma and urine selectively and accurately. The specificity and sensitivity were improved greatly over previous methods. We demonstrated the applicability of the method by analyzing PEITC in

plasma samples obtained in a preliminary clinical pharmacokinetic study of PEITC in four healthy volunteers following watercress consumption.

**Materials and Methods**

*Materials*

PEITC (99.9%), thiophosgene, ammonia (2M in 2-propanol) and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). 2-Phenylethyl-1,1,2,2-$^2H_4$-amine (99.3% atom %D) was purchased from CDN isotopes (Quebec, Canada). PEITC-NAC was kindly provided by Dr. Fung-Lung Chung (American Health Foundation, Valhalla, NY). Watercress was purchased from a local grocery store (Wegmans, Buffalo, NY). All the solvents were HPLC grade and were purchased from Fisher Scientific (Springfield, NJ).

*Synthesis of 1,1,2,2-$^2H_4$-PEITC:*

1,1,2,2-$^2H_4$-PEITC was synthesized following previously reported procedure [13]. The purity was >99% by HPLC and non-deuterated PEITC was not detected by mass spectrometry.

*Preparation of standard solutions*

A working stock solution of 25 μM PEITC in acetonitrile was prepared from a stock solution of 3 mM PEITC in acetonitrile. Calibration standards were prepared by appropriate dilution of PEITC to concentrations of 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000, 1500, and 2000 nM in HPLC water containing 300 nM of 1,1,2,2-$^2H_4$-PEITC as internal standard. Standard solutions for precision and accuracy determinations were prepared at PEITC concentrations of 20, 500, and 1500 nM in HPLC water. Recovery samples for human plasma and urine samples were prepared by adding the PEITC aqueous solutions to a plasma or urine sample at three levels (50, 500, and 1500 nM).

*Clinical specimen collection and preparation*

Four healthy volunteers (2 Caucasians and 2 Asians), ages 21 years and older, were recruited from students and staff at the University at Buffalo. Informed consent was obtained from each subject. Subjects were not allowed to have taken any medications or any cruciferous vegetables (such as watercress, broccoli, cauliflower, brussel sprouts, radishes, turnips or cabbage), condiments (such as horseradish, mustard or soy sauce), or herbal products for 3 days prior to the study, and did not eat or drink anything, except water, after midnight before the study day. Subjects ingested watercress at a dose of 100 grams on the study day. Blood samples were collected using venipuncture with heparinized sterile glass tubes. Approximately 5 ml of blood was collected prior to the ingestion of watercress, and then repeated samples were collected at 7.5, 15, 30, and 45 minutes, and at 1, 1.5, 2, 3, 4, 6, 8, and 24 hours after ingestion. A urine sample was collected one day prior to the study and on the study day at intervals of 0-1, 1-2, 2-4, 4-6, 6-8, 8-12, and 12-24 hours. Subjects had free access to water, but not to food, until 3 hours after the ingestion of watercress. No cruciferous vegetables or condiments (as listed above) were allowed during the study period.

Blood samples were collected into glass tubes containing heparin and centrifuged at 1500 *g* for 10 min. The plasma was transferred into polyethylene tubes and kept frozen at –80°C until analysis. Urine samples were collected into polyethylene tubes and kept frozen at –80°C until analysis.

*Sample extraction and derivatization*

An aliquot of 0.5 ml of human plasma or urine sample was transferred into a 5 ml glass tube and spiked with 6 µl of 25 µM (equivalent to 300 nM) IS. One ml n-hexane was added and gently vortexed for 20 s. After centrifugation at 1000 *g* for 3 min, the hexane phase was

removed and placed in a 10 ml glass screw top tube. A second extraction was repeated; two hexane extracts were combined and 2 ml of ammonia (2M in 2-propanol) was added for derivatization. The mixture was incubated on a shaking bed and allowed to react for 6 h at room temperature. The mixture was then dried under a $N_2$ stream at 50°C and reconstituted with 50 µl of acetonitrile/$H_2O$ (3:2, v/v) by vortex mixing. The reconstituted sample was transferred into a 200 µl autosampler vial insert for analysis by LC/MS/MS.

*LC/MS/MS*

The LC/MS/MS system consisted of a PE SCIEX API 3000 triple-quadruple tandem mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a heated nebulizer interface, a series 2000 Perkin-Elmer pump, and a series 2000 Perkin-Elmer autosampler (Shelton, CT). Separation was carried out on a $C_{18}$ (particle size 5 µm; 150×4.6mm) column (Alltech, Deerfield, IL) and the mobile phase consisted of acetonitrile/5mM formic acid (60:40, v/v). The flow rate was 1 ml/min and the injection volume was 5 µl. The mass spectrometer was operated in positive ionization mode. The parameter settings for nebulizer, curtain and collision gasses were 8, 10 and 5 units. The declustering, focusing and entrance potential were 20, 350 and –10 V, respectively. Multiple reaction monitoring (MRM) of MS/MS was used for specific detection of the derivatives of PEITC and IS by measuring the characteristic ion transition of *m/z* 181 (parent ion) to *m/z* 105 (product ion), and *m/z* 185 (parent ion) to *m/z* 109 (product ion), respectively.

*Calibration and validation*

Calibration was performed by an internal standard method. The integration was processed on Analyst software (Applied Biosystems, Foster City, CA), and calibration curves were obtained by plotting extracting ion current (XIC) peak area ratios of analyte/internal standard vs.

concentrations. Standard curves were run on each analysis day and the coefficient of determination $r^2$ was used to judge linearity.

Intra- and inter-day precision and accuracy as well as recovery from plasma and urine samples were assessed through triplicate analysis of same samples containing known amounts of PEITC, with 3 samples per concentration level. Precision was estimated as CV% of the mean of all the determinations at each concentration level. Accuracy was determined by comparing the calculated concentrations to the known concentrations. Recovery was calculated by comparing the determined amounts for extracted blood or urine samples with the known amounts added. The limit of detection (LOD) was assessed as the PEITC concentration at a signal-to-noise of 3:1. The lower limit of quantitation (LLOQ) was defined as the PEITC concentration yielding a mean assayed concentration within 20% of the known concentration as well as a precision with CV% less than 20%.

*Pharmacokinetic Analysis*

The plasma concentrations over time were evaluated and the pharmacokinetic parameters were estimated by non-compartmental and compartmental model analysis using WinNonlin Professional Edition Version 2.1 (Pharsight, Mountainview, CA). The dose was determined to be 25 mg PEITC, based on the calculation that 30 mg watercress contained 7.6 mg PEITC [14]. Apparent absorption rate constant (ka), clearance (Cl/F) and volume of distribution (V/F) were fitted. The maximal plasma concentration ($C_{max}$) and the time to reach $C_{max}$ ($t_{max}$) were determined directly from the plasma concentation vs. time profile. The elimination half-life ($t_{1/2}$) was estimated from the terminal slope of the plasma concentration profile. Renal clearance ($Cl_R$) was calculated by dividing the total amount of PEITC excreted in the urine ($A_e$) by the area under the plasma concentration vs. time curve (AUC).

## Results and Discussion

*Selective extraction of PEITC from the matrix*

The selective extraction of PEITC from the biological matrix is necessary to avoid interference from other components, especially PEITC metabolites. We separated PEITC from polar substances present in plasma or urine samples by extraction with hexane, a nonpolar solvent. PEITC-NAC, the major metabolite of PEITC, and other potential metabolites generated along the mercapturic acid pathway, are polar compounds that would not be extracted into hexane. Analysis of the hexane phase after extraction of an aqueous solution of PEITC-NAC revealed that no PEITC-NAC was present (data not shown). Hexane extraction was conducted twice to ensure the majority of the analyte was extracted taking account of its low amount in clinical samples. In addition, increased extraction times did not show significant improvement of the assay sensitivity.

*Derivatiztion of PEITC to phenethylthiourea*

After the hexane extraction, an excess amount of ammonia (in 2-propanol) was added to the organic phase. The reaction of PEITC and ammonia occurs at room temperature efficiently. LC/MS is of restricted usage if analyzing PEITC directly primarily due to the low ionization capability of the compound. On the other hand, ITCs are reactive electrophilic compounds that easily react with O-, S-, and N-nucleophiles. Due to good nucleophilicity of amines, we initially investigated the use of phenethylamine to react with PEITC. The UV absorptivity of the derivative was substantially higher than that of PEITC, and so HPLC could possibly be used to analyze clinical samples. However, the excess amount of phenethylamine was hard to remove and the chromatographic peak of phenethylamine tailed and interfered with the peak of the derivative. Since volatile amines would not have this problem because of their easier removal,

we chose to investigate the use of ammonia for the reaction with PEITC. We found that this reaction was highly efficient at room temperature in aqueous solution. However, PEITC was present in the hexane phase after extraction, resulting in inefficient reaction with ammonia aqueous solution due to the immiscibility of the two phases. We found that 2-propanol mixed thoroughly with hexane; therefore, ammonia in 2-propanol solution was utilized and the reaction was complete within 6-8 hours. The product, phenethylthiourea, is polar and non-volatile; hence it represents a good substrate for LC/MS analysis, as shown by the mass spectra (Fig. 3). Additionally, the loss of PEITC during sample extraction and $N_2$ evaporation, due to its volatility, is avoided.

Unlike the cyclocondensation derivatization, the thiourea derivative is unique for each ITC (Fig. 2). Therefore, the interference from other ITCs was prevented by designation of the ions specifically from the PEITC derivative in MRM and also by separation of the eluates by HPLC. It is highly unlikely that another compound would form a derivative that would have the same molecular and fragment ions as well as the same retention time as that of PEITC. Although PEITC-NAC and some other metabolites may also form phenethylthiourea after reaction with ammonia, they were removed during extraction and would not be present in hexane extracts for derivatization. Thus, the interference from PEITC metabolites was avoided. As a consequence, this analytical approach allowed specific detection of PEITC with minimal interference from other ITCs, the metabolites, and DTCs.

*LC/MS/MS*

The full scan and product ion mass spectrum of the PEITC derivative, phenethylthiourea, is presented in Fig. 3A and 3B respectively. The major product ion from the parent ion *m/z* 181

($[M+H]^+$) was *m/z* 105 (Fig. 3B) due to the loss of the thiourea fragment. The analyses were performed using MRM pairs of *m/z* 181→105 for the analyte.

The retention time of the thiourea derivative of PEITC was typically 2.2 min (Fig. 4). The noise level was low with the intensity lower than 200 counts per second (cps) and no interfering peaks at the retention time of 2.2 min were found on analysis of the plasma and urine samples of four subjects. Due to high specificity of MS/MS, the thiourea derivative of PEITC was unambiguously identified by its MRM pairs (181→105), even if it co-eluted with other derivatized ITCs.

*Validation of the assay*

Based on the PEITC level present in clinical samples, quantitation was typically performed at nanomolar concentrations. Validation was performed in terms of LOD, LLOQ, linearity, intra-day and inter-day precisions and accuracies, and recoveries in human plasma and urine. LOD and LLOQ are important characteristics for assay sensitivity when biological matrixes are analyzed, particularly when the analyte is present at low or trace concentrations. Based on six replicates assayed on three different occasions, the LOD was 2 nM and the LLOQ was 7.8 nM. Besides much better selectivity, our method has improved sensitivity than the modified cyclocondensation method for clinical purposes (total ITC has LOD and LLOQ of 20 nM and 98 nM respectively) [10].

Calibration curves of PEITC were linear over the concentration range of 7.8 to 2000 nM ($r^2$ values were typically greater than 0.995) (Fig. 5). During quantitative analysis, variation in signal response due to external causes, such as contaminants in the ion source and ionization efficiency, are corrected by the internal standard that ionizes simultaneously with the analyte. Since the internal standard we used is an isotopically labeled analogue, the ionization and

fragmentation behavior is exactly identical to that of the analyte; therefore, we were able to obtain good linearity.

For precision, %CV values for PEITC were less than 5% for intra-day analysis, and less than 10% for inter-day analysis (Table 1). The intra- and inter-day percent accuracy for PEITC was 101-105% and 102-119%, respectively (Table 1). The recoveries of PEITC from spiked human plasma and urine samples were within 14% and 4% of theoretical values (Table 2). These data demonstrated that the interference from the metabolites, other ITCs or DTCs was not detectable and the method is able to quantitate PEITC in plasma and urine accurately and specifically.

*Clinical sample analysis*

We detected and quantitated PEITC in most of the plasma and urine samples from four healthy volunteers after ingestion of 100 g watercress (approximately 25 g PEITC) and the plasma concentration vs. time profile is shown in Fig. 8. It has been reported that little or no PEITC was detected in most of the plasma samples from volunteers who ingested 40 g of watercress (releasing 6-12 mg PEITC) and from subjects after 40 mg oral PEITC administration [10]. Additionally, PEITC was not detected in urine samples in a previous study of PEITC following watercress consumption using HPLC [14]. Therefore, our method showed improved sensitivity compared to previous studies.

*Pharmacokinetics in humans after watercress consumption*

The pharmacokinetic parameters of the four subjects are shown in Table 3. The plasma concentration for the four subjects was fitted to a one-compartment model based on the criteria of goodness-of-fit (Fig. 6). However, we do not preclude the possibility of fitting to a multi-compartment model due to our limited data collection between 8-24 h after watercress ingestion.

The endogenous plasma total ITC levels in plasma samples obtained from subjects without food restriction were 413 (± 193) nM (mean (SD), n = 23) [10]. When we analyzed plasma samples taken before the initiation of the study but after 3 days of dietary restriction, we still measured low concentrations of PEITC in the subjects (19.3 ± 14.3 nM, mean ± SD, n = 4). Although we provided subjects with a list of restricted food items that are known to contain PEITC, it is likely there are additional dietary sources of PEITC. Consequently, if human plasma is used as the matrix to perform calibration curves, baseline levels of PEITC need to be taken into account. Although the error may be minor when samples contain high concentrations, it would be significant for samples with low PEITC concentrations that are close to the baseline level. This may affect determination of some pharmacokinetic parameters. For example, elimination half-life is determined at later time points when PEITC concentrations are low and therefore may be biased by baseline levels of PEITC. Hence, we prepared different PEITC standards in water and thus to measure the absolute amount of PEITC in each sample. We then subtract out the baseline level from each sample measurement. The recovery from plasma and urine indicated that the assay is able to quantitate PEITC in those matrixes accurately.

The apparent absorption rate constant ($k_a$) for the four subjects was 1.3 (± 0.3) $h^{-1}$, determined based on one-compartment model. This is a complex parameter because it represents not only the absorption rate but also the hydrolysis rate of gluconasturtiin to PEITC by the microflora present in gastrointestinal tract. The $t_{max}$ value was 2.6 (± 1.1) hours, indicating that plasma concentrations peak relatively rapidly. This data, taken together with the $k_a$ value, suggest that the absorption of PEITC is relatively fast. Although PEITC has a low polarity and a low molecular weight and one might anticipate rapid absorption, we cannot exclude the possibility of flip-flop kinetics (absorption being slower than elimination) in this study. Total ITC (at least

including PEITC and its conjugates) peaked at 4.6 (± 0.7) hours in three subjects after oral dosage of PEITC in capsules [10].

The elimination half-life $t_{1/2}$ of PEITC in our study was 4.9 ± 1.1 hours, while total ITC had a $t_{1/2}$ of 3.7 ± 1.3 hours [10]. In addition to the difference in dosage form and chemical forms analyzed, the discrepancies can also result from inter-individual variability and limited subject numbers. It has been known that glutathione-S-transferase M1 (GSTM1) and GSTT1, the two major GSTs responsible for PEITC metabolism in humans exhibit polymorphisms in the population due to homozygous deletion of the genes [15]. About 50% of the population have GSTM1 null type; while 12-16% in Germans and English, and 60-64% Chinese and Koreans have GSTT1 null type [16]. Consequently, presence or absence of the enzymes due to different genotypes can affect the metabolism greatly resulting in variable $t_{1/2}$ and $t_{max}$ of PEITC, especially when the subject number is small.

**Summary**

In conclusion, a novel LC/MS/MS procedure with high sensitivity and specificity was developed and validated to analyze PEITC in human plasma and urine samples. The method consists of sample extraction by hexane, followed by its ammonia derivatization to thiourea, chromatographic separation on a $C_{18}$ column and then detection in MRM mode. High selectivity was achieved by selective extraction of the analyte from the biological matrices by hexane, ammonia derivatization to thioureas that maintains the chemical identity of different ITC, and the combination of HPLC with specific MRM of a characteristic transition of the analyte derivative. The use of a stable isotopically labeled internal standard ensured the accuracy of quantitation and eliminated a matrix effect. To our knowledge, this is the first assay with the specificity and sensitivity to determine unchanged PEITC in biological samples. Our preliminary clinical study

demonstrated that the method was able to characterize the pharmacokinetics of PEITC in human subjects after watercress ingestion and therefore will be valuable for further clinical investigations.

**Acknowledgements**

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* Corresponding author. Fax: 716-645-3693

*Email address:* memorris@buffalo.edu (M.E. Morris)

[1] *Abbreviations used:* ITC, isothiocyanate; PEITC, phenethyl isothiocyanate; LC/MS/MS, liquid chromatography-tandem mass spectrometry; GC/MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; GC, gas chromatography; DTC, dithiocarbamate; PEITC-NAC, PEITC-N-acetylcysteine; MRM, multiple reaction monitoring; IS, internal standard; XIC, extracting ion current; LOD, limit of detection; LLOQ, lower limit of quantitation; ka, absorption rate constant; Cl, clearance; V, volume of distribution; F, bioavailability; $C_{max}$, maximal plasma concentration; $t_{max}$, time to reach $C_{max}$; $t_{1/2}$, elimination half-life; $Cl_R$, renal clearance; $A_e$, amount excreted in the urine; AUC, area under the plasma concentration vs. time curve; GST, glutathione-S-transferase.

Fig. 1. Structures of PEITC and PEITC-NAC.

Fig. 2. Derivatization schemes for ITCs. (A) Cyclocondensation reaction of ITCs and dithiocarbamates with 1,2-benzenedithiol to yield 1,3-benzenedithiol-2-thione; (B) Reaction of PEITC with ammonia to yield phenethylthiourea.

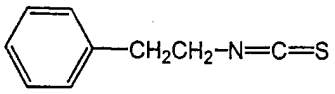
Fig. 3. The full scan (A) and product ion (B) mass spectrum of phenethylthiourea, the derivative of PEITC (*m/z* 181 is the molecular ion ($[M+H]^+$) and *m/z* 105.0 is the major product ion).

Fig. 4. The chromatograms of LC/MS/MS analysis of human plasma (obtained during a clinical study) containing analyte (A) and internal standard (spiked) (B), and human urine (obtained during a clinical study) containing analyte (C) and internal standard (spiked) (D). The derivative of PEITC, phenethylthiourea, has a retention time of 2.2 min.

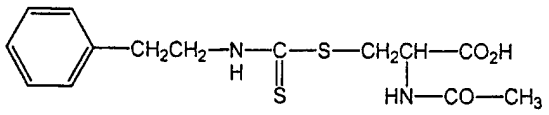
Fig. 5. A representative calibration curve for PEITC by LC/MS/MS analysis. Each point is the average of peak area ratios of duplicate injections for the derivative phenethylthiourea.

Fig. 6. Plasma concentration versus time profile of PEITC in humans following the consumption of 100 g watercress. Data are expressed as mean ± SD, n = 4; closed circles represent the measured concentration and the line represents the predicted concentration fitted by WinNonlin.

Fig. 1



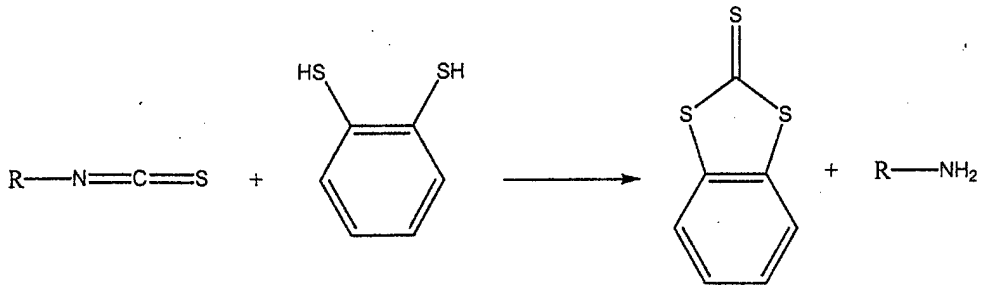

PEITC




PEITC-NAC

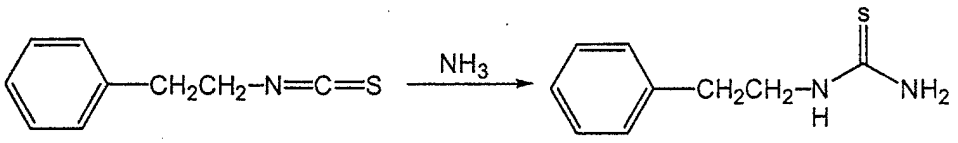
Fig. 2

(A)




ITC 1,2-benzenedithiol 1,3-benzenedithiol-2-thione

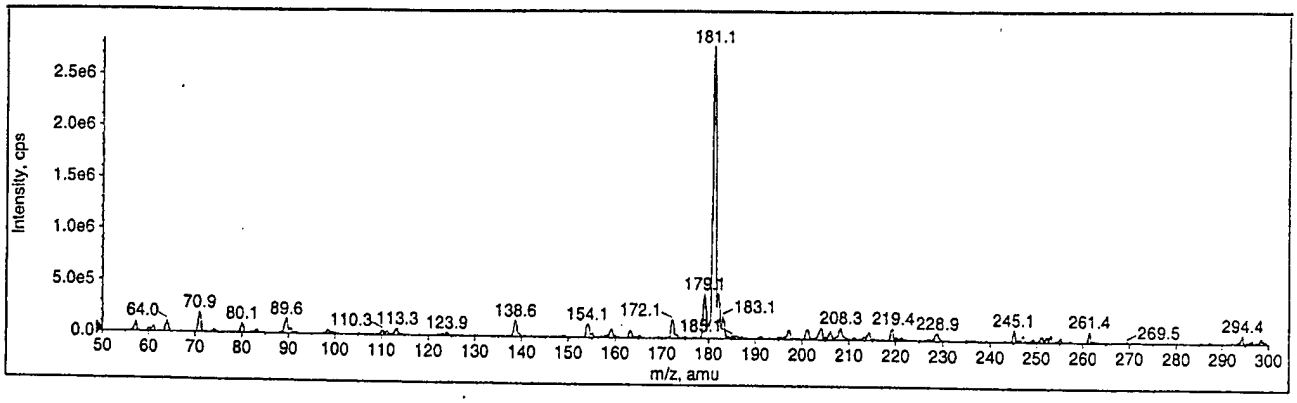
(B)




PEITC phenethyl thiourea

Fig. 3

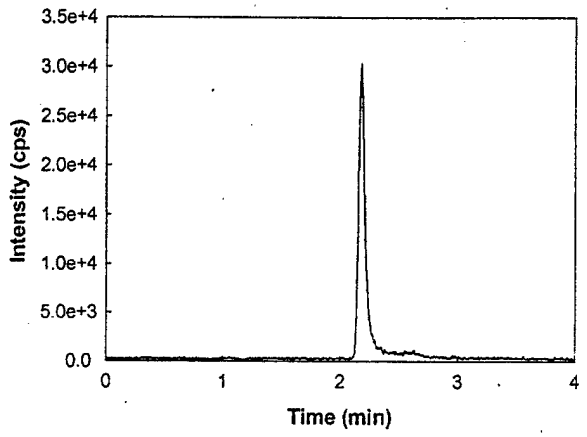
(A)


Intensity, cps
2.5e6
2.0e6
1.5e6
1.0e6
5.0e5
0.0
181.1
179.1
64.0
70.9
80.1
89.6
110.3
113.3
123.9
138.6
154.1
172.1
183.1
208.3
219.4
228.9
245.1
261.4
269.5
294.4
50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300
m/z, amu

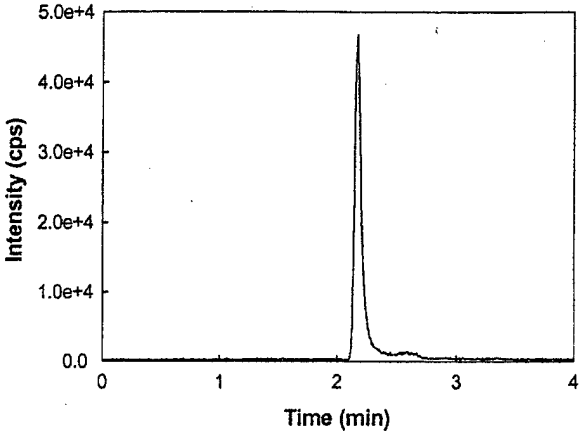
(B)

Fig. 4

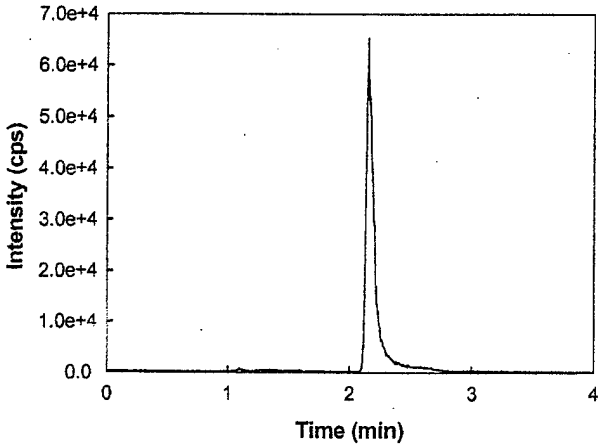
(A)


3.5e+4
3.0e+4
2.5e+4
2.0e+4
1.5e+4
1.0e+4
5.0e+3
0.0
Intensity (cps)
0
1
2
3
4
Time (min)

(B)


5.0e+4
4.0e+4
3.0e+4
2.0e+4
1.0e+4
0.0
Intensity (cps)
0
1
2
3
4
Time (min)

(C)


7.0e+4
6.0e+4
5.0e+4
4.0e+4
3.0e+4
2.0e+4
1.0e+4
0.0
Intensity (cps)
0
1
2
3
4
Time (min)

(D)

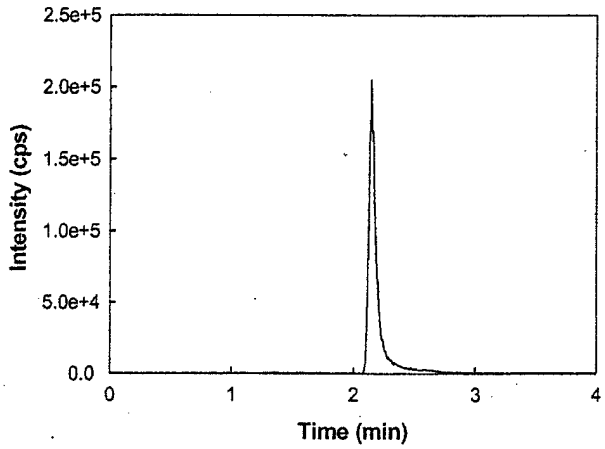

2.5e+5
2.0e+5
1.5e+5
1.0e+5
5.0e+4
0.0
Intensity (cps)
0
1
2
3
4
Time (min)

Fig. 5

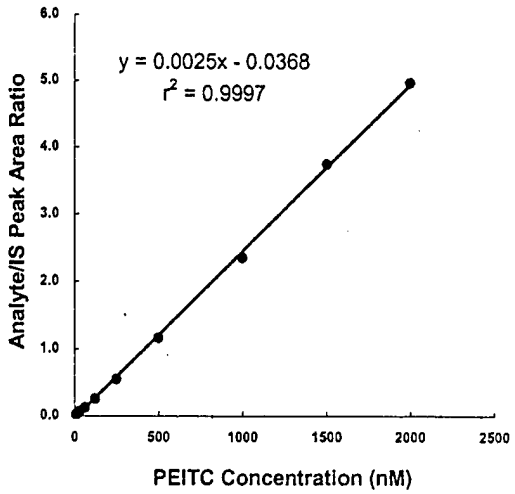

y = 0.0025x - 0.0368
r2 = 0.9997
Analyte/IS Peak Area Ratio
PEITC Concentration (nM)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
500
1000
1500
2000
2500

Fig. 6

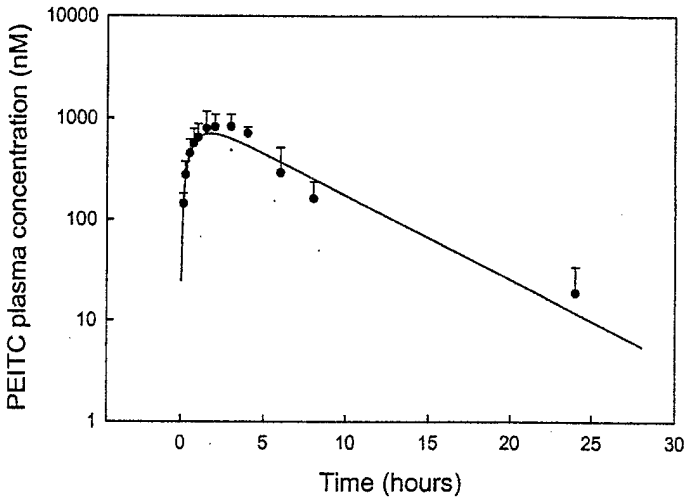

PEITC plasma concentration (nM)
10000
1000
100
10
1
0
5
10
15
20
25
30
Time (hours)

Table 1
Accuracy and precision of the LC/MS/MS assay of PEITC

| | Expected Concentration (nM) | Mean measured Concentration (nM) | SD | Precision (CV%) | Accuracy (%) |
|---|---|---|---|---|---|
| Intra-day | 20 | 20.6 | 1.0 | 4.8 | 103.1 |
| | 500 | 505.0 | 6.5 | 1.3 | 101.0 |
| | 1500 | 1563.6 | 48.8 | 3.1 | 104.2 |
| Inter-day | 20 | 23.7 | 2.2 | 9.3 | 118.6 |
| | 500 | 533.4 | 31.1 | 5.8 | 106.7 |
| | 1500 | 1542.6 | 96.1 | 6.2 | 102.8 |

Table 2
Recovery of PEITC in human plasma and urine

| Concentration | Plasma | | | Urine | | |
|---|---|---|---|---|---|---|
| (nM) | Recovery (%) | SD | CV% | Recovery (%) | SD | CV% |
| 50 | 113.5 | 5.2 | 4.6 | 98.3 | 8.7 | 8.8 |
| 500 | 100.3 | 2.1 | 2.1 | 103.9 | 3.4 | 3.3 |
| 1500 | 103.0 | 2.4 | 2.3 | 102.1 | 9.0 | 8.8 |

Table 3
Pharmacokinetic parameters of PEITC in humans after ingestion of 100 g watercress

| | Subject 1 | Subject 2 | Subject 3 | Subject 4 | Mean | SD | CV% |
|---|---|---|---|---|---|---|---|
| $C_{max}$ (nM) | 753.4 | 1132.3 | 673.4 | 1155.0 | 928.5 | 250.7 | 27.0 |
| $t_{max}$ (h) | 2.0 | 3.0 | 4.0 | 1.5 | 2.6 | 1.1 | 42.2 |
| $k_a$ ($h^{-1}$) | 1.5 | 1.0 | 1.0 | 1.5 | 1.3 | 0.3 | 22.1 |
| $t_{1/2}$ (h) | 5.9 | 5.7 | 3.6 | 4.4 | 4.9 | 1.1 | 22.8 |
| Cl/F (L/h) | 32.0 | 17.6 | 43.1 | 25.4 | 29.5 | 10.8 | 36.6 |
| V/F (L) | 193.8 | 110.4 | 196.1 | 117.6 | 154.5 | 46.8 | 30.3 |
| $CL_R$ (L/h) | | | 0.21 | 0.025 | | | |

**Determination of Alpha-Naphthylisothiocyanate and Its Metabolite Alpha-Naphthylamine in Rat Plasma and Urine by High-Performance Liquid Chromatographic Assay.**

Ke Hu* and Marilyn E. Morris. *Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, State University of New York at Buffalo, Buffalo, NY 14260, USA*

**Abstract**

**Purpose:** To develop an HPLC assay for determination of alpha-naphthylisothiocyanate (1-NITC), a potential P-glycoprotein modulator, and its metabolite alpha-naphthylamine (1-NA) in rat plasma and urine. **Methods:** The chromatographic analysis was carried out using a Partisphere C-18 5 μm column (125 × 4.6 mm) with a mobile phase of acetonitrile (ACN):$H_2O$ (70:30, v/v) and UV detection at 305 nm. Naphthalene was used as the internal standard. Stability studies were performed at varying temperatures and pH values. Rat plasma and urine samples were analyzed for 1-NITC and 1-NA, following i.v. administration of 1-NITC to rat. **Results:** 1-NITC and 1-NA had retention time 5.9 and 2.2 min, respectively. The lower limit of quantitation in plasma and urine samples were 10 and 30 ng/ml for 1-NITC, and 30 and 100 ng/ml for 1-NA. The within-day and between-day accuracy and precision were 95-106% and 93-100% for 1-NITC in plasma. For 1-NA in urine, the within- and between-day accuracy and precision were 96-106% and 97-99%. The ACN extraction was efficient for both plasma and urine samples based on recovery of 93-97% for 1-NITC, and of 95-110% for 1-NA. 1-NITC was stable at all tested temperatures in ACN, and at -20 and -80°C in plasma, urine, and ACN extracts of plasma and urine. 1-NA was stable in all tested matrix. The assay was used to analyze plasma and urine samples following administration of an i.v. dose of 25 mg/kg to rat. 1-NITC and 1-NA were detected in plasma and urine, respectively. Based on noncompartmental analysis by WinNonLin 2.0, the fitted parameters clearance (CL 2.07 l/kg/h), volume of distribution (V 14.3 l/kg), and half life ($t_{1/2}$ 4.76 h) were determined for 1-NITC. **Conclusion:** A rapid and sensitive HPLC assay has been developed for determination of 1-NITC and its metabolite 1-NA in rat plasma and urine for future pharmacokinetic and pharmacodynamic studies.

# EFFECT OF ORGANIC ISOTHIOCYANATES ON THE P-GLYCOPROTEIN AND MRP1-MEDIATED TRANSPORT OF DAUNOMYCIN AND VINBLASTINE

**Elaine Tseng, Amrita Kamath and Marilyn E. Morris**

Dept. of Pharmaceutical Sciences, University at Buffalo,
State University of New York

Memorris@acsu.buffalo.edu

Purpose. Organic isothiocyanates (ITCs) (mustard oils) are non-nutrient components present in the diet, especially in cruciferous vegetables. The purpose of this investigation was to examine the effect of ITCs on P-glycoprotein (P-gp)- and Multidrug Resistance-Associated Protein (MRP1)-mediated transport in multidrug resistant (MDR) human cancer cell lines.

Methods. The direct effect of organic isothiocyanates on the 2-hour cellular accumulation of daunomycin (DNM) and vinblastine (VBL), substrates for both P-gp and MRP1, were measured in sensitive and resistant MCF-7 cells and in PANC-1 cells. Resistant MCF-7 cells (MCF-7/ADR) overexpress P-gp while PANC-1 cells overexpress MRP1. The following compounds were evaluated: allyl-, benzyl-(BITC), hexyl-, phenethyl-(PEITC), phenyl-, 1-naphthyl-(NITC), phenylhexyl-, phenylpropyl-, phenylbutyl- isothiocyanate, sulforaphane, erucin and erysolin.

Results. NITC significantly increased the accumulation of DNM and VBL in both resistant cell lines, but had no effect on DNM accumulation in sensitive MCF-7 cells. VBL accumulation in resistant MCF-7 cells was increased 40-fold by NITC, while that in PANC-1 cells was increased 5.5-fold. Significant effects on the accumulation of DNM and VBL in resistant MCF-7 cells were also observed with BITC, while PEITC, erysolin, phenylhexyl-ITC and phenylbutyl-ITC increased the accumulation of DNM and/or VBL in PANC-1 cells. Overall, the inhibitory activities of these compounds in MCF-7 cells and PANC-1 cells were significantly correlated ($r2 = 0.77$ and 0.86 for DNM and VBL, respectively). Significant effects on accumulation were generally observed with the ITCs at 50 mM concentrations, but not at 10 mM concentrations.

Conclusions. One strategy to enhance the effectiveness of cancer chemotherapy is to reverse the MDR phenomena. Our results indicate that certain dietary ITCs inhibit the P-gp- and the MRP1-mediated efflux of DNM and VBL in MDR cancer cells, and suggest the potential for diet-drug interactions.

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**Effects of Benzyl-, Phenethyl- and alpha-Naphthyl isothiocyanates on P-glycoprotein- and MRP1-Mediated Transport of Daunomycin.** Ke Hu* and Marilyn E. Morris. *Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, State University of New York at Buffalo, Buffalo, NY 14260, USA*

**Abstract**

**Purpose:** To evaluate the effects of isothiocyanates (ITCs) on P-glycoprotein (P-gp)- and Multidrug Resistance Protein (MRP1)-mediated efflux of daunomycin (DNM), determine whether ITCs are substrates of P-gp and/or MRP1, and elucidate the mechanism(s) involved in the inhibition of transport.

**Methods:** The effects of benzyl- (BITC), phenethyl- (PEITC) and alpha-naphthyl isothiocyanates (1-NITC) on the 2-h accumulation of DNM in human breast cancer MCF-7 and MDA435/LCC6, colonic adenocarcinoma Caco-2, and pancreatic adenocarcinoma PANC-1 cells were evaluated. Verapamil (VRP, P-gp inhibitor) and MK571 (MRP1 inhibitor) were used as positive controls. $^{14}$C-PEITC was used for substrate studies in MDA435/LCC6, MDA435/LCC6MDR1, Caco-2 and PANC-1 cells in the absence and presence of VRP or MK571. Cellular concentrations of glutathione (GSH) and activities of glutathione-S-transferase (GST) were measured after 2- and 24-h drug treatment in PANC-1 and Caco-2 cells.

**Results:** BITC, PEITC and 1-NITC significantly increased the accumulation of DNM in MCF-7/ADR, Caco-2 (except for 1-NITC), and PANC-1 cells. The uptake of PEITC was not changed by VRP in Caco-2, MDA435/LCC6 and MDA435/LCC6MDR1, but significantly increased by MK571 in PANC-1 cells. Cellular GSH was profoundly depleted in PANC-1 and Caco-2 cells by BITC and PEITC (6-100-fold), but not by 1-NITC. GST activities were not changed with treatment.

**Conclusion:** ITC group, aryl rings and the length of alkyl chains play key roles in reversal activity, besides Log P. PEITC is a substrate of MRP1 rather than P-gp. It is probably that the increased accumulation of DNM by BITC and PEITC is due to the dramatic depletion of cellular GSH (co-substrate for DNM efflux) and the competitive binding of glutathione conjugates (ITC-SGs) to D-site of MRP1 with DNM. The mechanism of 1-NITC has not been known. The inactivity of 1-NITC to increase DNM uptake in Caco-2 cells is most likely due to its extensive metabolism by cytochrome P450 1A1.

**Pharmacokinetics of α-Naphthylisothiocyanate in Rats.** Ke Hu* and Marilyn E. Morris.
*Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, State University of New York at Buffalo, Buffalo, NY 14260, USA*

**Abstract**

**Purpose:** To investigate pharmacokinetics of α-naphthylisothiocyanate (1-NITC) in rats.

**Methods:** Pharmacokinetic studies of 1-NITC were performed with four doses of 10, 25, 50 and 75 mg/kg to Sprague-Dawley female rats (n = 4 for each group; body weight 200-250 g) via i.v. administration. Blood samples (250 μl each) were collected from the jugular vein at 5, 10, 20, 30 min, 1, 2, 4, 6, 9, 12, 24, 36 and 48 h (36 and 48 h for 50 and 75 mg/kg groups). The concentrations of 1-NITC in plasma were determined by HPLC assay with C18 column (125 × 4.6 mm i.d., 5 μm), a mobile phase consisting of ACN-$H_2O$ (70:30, v/v), flow rate at 1.0 ml/min, and the detection wavelength at UV 305 nm. The data were simultaneously fitted using ADAPT II software.

**Results:** 1-NITC exhibited nonlinear Michaelis Menten disposition and data were characterized with a two compartment open model. Parameters were estimated as: maximium velocity ($V_{max}$), 2.13 ± 0.20 mg/h/kg; Michaelis Menten constant ($K_m$), 0.51 ± 0.13 mg/L; first order rate constant from central to tissue compartment ($k_{12}$), 1.10 ± 0.15 $h^{-1}$; first order rate constant from tissue to central compartment ($k_{21}$), 0.32 ± 0.04 $h^{-1}$; volume of central compartment ($V_C$), 3.37 ± 0.21 L/kg; volume of tissue compartment ($V_T$), 11.72 ± 0.85 L/kg.

**Conclusion:** 1-NITC demonstrated nonlinear pharmacokinetics via i.v. administration. These results will be used to support the application of 1-NITC in combination with anticancer drug doxorubicin (DOX) to reverse P-glycoprotein (P-gp)- and Multidrug Resistance Protein 1 (MRP1)-mediated multidrug-resistance (MDR).