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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
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. The first two specific aims of our proposal are: 1) To determine the concentration-dependent effects of benzyl ITC (BITC), phenethyl ITC (PEITC) and naphthyl ITC (NITC) on the accumulation of ^{3}H-daunomycin (DNM) in sensitive and resistant human breast cancer cell lines, and 2) To evaluate the pharmacokinetics and toxicity of NITC, PEITC and BITC in the rat following oral and intravenous administration. NITC, PEITC and BITC significantly increased the cellular accumulation of DNM and vinblastine (VBL) in resistant human breast cancer MCF-7 cells at 20-100 μM concentrations, without affecting accumulation in MCF-7 sensitive cells. Cytotoxicity studies revealed that PEITC and BITC inhibited the growth of both the MCF-7 and normal mammary MCF-12A cell lines. Assays to determine PEITC, BITC and NITC in biological fluids were developed, and stability studies demonstrated limited stability of NITC in biological fluids at RT, while PEITC and BITC degraded with half-lives of 36 and 40 h, respectively, at pH 7.4. Characterization of the pharmacokinetics of NITC in rats revealed a high clearance (2.29±0.81 L/kg/h) and large volume of distribution (16.8±3.6 L/kg). The ITCs may represent a new class of inhibitors of MDR in breast cancer.

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Annual Research Report June 1, 2001-May 31, 2002

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

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 was addressed in the 2000-01 Annual Report. *The manuscript has been accepted for publication in Pharmaceutical Research (attached in Appendix A).* Cytotoxicity studies were also performed examining the direct cytotoxicity of BITC, PEITC and NITC in human breast cancer MCF-7 and human mammary epithelial MCF-12A cells.

Cytotoxicity Studies. Studies examining the effect of PEITC, BITC and NITC on the cytotoxicity of doxorubicin were reported in the 2000-01 Annual Report. In those studies, we observed toxicity at the higher concentrations of ITCs following 48-hour incubation periods. Therefore, studies were conducted to evaluate the direct cytotoxicity of the three ITCs. *A manuscript (to be submitted) reporting the direct cytotoxicity of these ITCs in the breast cancer cell line MCF-7 and the normal mammary cell line MCF-12A is attached in Appendix B.* Our results are summarized below:
Purpose. The purpose of this investigation was to examine the cytotoxicity of α-naphthyl isothiocyanate (NITC), benzyl isothiocyanate (BITC) and β-phenylethyl isothiocyanate (PEITC) in a human breast cancer MCF-7 and a human mammary epithelium MCF-12A cell lines.
Methods. The cytotoxicity of PEITC, BITC, NITC, in concentrations varying from 0.1μM to 50μM and that of daunomycin (DNM) and vinblastine (VBL) were examined following various exposure times, up to 48 hours, in the cultured cells. Cell growth was determined by a sulforhodamine B assay.
Results. The IC_{50} values of DNM and VBL in MCF-7/Adr cells were 40.10 ± 1.74 μM and 9.99 ± 0.803 μM, respectively, following a 2-hour exposure and decreased to 7.12 ± 0.42 μM and 0.106 ± 0.004 μM following a 48-hour exposure. The corresponding values for BITC and PEITC are 10.37 ± 0.31μM and 13.98 ± 0.54 μM for a 2-hour exposure and 5.95 ± 0.10μM and 7.32 ± 0.25 μM for a 48-hour exposure. IC_{50} values for NITC in MCF-7/Adr cells could not be determined since there was limited toxicity at concentrations up to 50 μM. In MCF-12A cells, the IC_{50} of DNM was not able to be determined due to the limited toxicity seen at concentrations up to 100μM at the 48-hour endpoint. Corresponding values for BITC, PEITC, and NITC were 8.07 ± 0.29 μM, 7.71 ± 0.074 μM and 33.63 ± 1.69 μM, respectively. The differences in IC_{50} between cancer and normal cells may be due to a number of factors in these two cell lines, including intrinsic differences in sensitivity to the cytotoxicity of these compounds, differences in free drug concentrations due to protein binding considerations as a result of differences in the protein content of media, differences in the metabolism of these compounds, or differences in the intracellular accumulation of these compounds in these two cell lines.
Conclusions. BITC and PEITC, which are present in cruciferous vegetables in mg quantities, can inhibit the growth of human breast cancer cells as well as normal human mammary

epithelium cells, at concentrations similar to the chemotherapeutic drug daunorubicin. This effect on cell growth may contribute to the cancer chemopreventive properties of ITCs by suppression of the growth of preclinical tumors, and/or may indicate a potential use of these compounds as chemotherapeutic agents in cancer treatment.

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. An alternative method is briefly described under Methods. The second compound to be synthesized will be radiolabeled NITC since this compound appears to be the most potent inhibitor of the three ITCs.
Time: Year 1 - Year 2

Overview:
^{14}C-PEITC was synthesized and purified. The use of LC/Ms/MS assays for the analysis of NITC, BITC and PEITC was evaluated. A GC/MS assay was developed for PEITC in order to provide increased sensitivity for the analysis of biological samples.

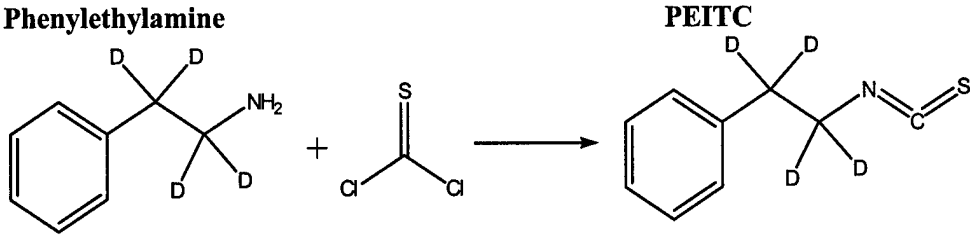
a) ^{14}C-PEITC was prepared by a three-step synthesis. The starting material was [^{14}C]KCN (25mCi), and the two intermediates were [^{14}C] benzylcyanate and [^{14}C]phenethylamine. The compound was purified using a silica column, and purity determined using NMR and HPLC (with fraction collection and radioactivity counting). *(Protocol is attached- Appendix C)*

b) Since the Department of Pharmaceutical Sciences at the University at Buffalo has obtained an LC/MS/MS instrument (PE Sciex API 3000), we investigated the use of this technique for the analysis of the ITCs. We had indicated in our original proposal that we would use LC/MS/MS if the instrumentation became available. We have developed an LC/MS/MS assay for PEITC mercapturic acid (reported in 2000-01 annual report). We have spent considerable time trying to develop LC/MS/MS assays for PEITC, BITC and NITC. Possible reasons for the lack of success of the LC/MS assay for PEITC and BITC include: 1) *Low molecular weight.* PEITC and BITC have molecular weights of 163.2 and 149.22, respectively. They fall into the noise range of LC/MS. 2) *High volatility.* PEITC is volatile and easily to evaporate under vaporization of mobile phase. Therefore, only trace amounts enter the mass spectrometer. 3) *Low polarity.* PEITC is not easily ionized by electrospray ionization (ESI) in LC/MS due to its low polarity. 4) *Low aqueous stability.*

We are developing a GC/MS assay for PEITC using a Hewlett Packard 5972 Mass selective detector with a 5890 Series II gas chromatograph, data station and 7673 auto sampler. This instrument is available through the Dept. of Chemistry at the University at Buffalo. The separation uses a 30m × .25mm capillary column HP-5 (5% phenyl methyl silicone), injection temperature of 250 °C, column temperature of 170 °C, and helium flow rate of 1ml/min. The mass spectrometric conditions are: electron ionization (EI) source and detector temperature 200 °C. Selected ion monitoring of fragments of m/z 163, 105 and 91 is used for analysis of PEITC.

β-phenylethyl-1,1,2,2-($^2H_4$) amine has been used to synthesize β-phenylethyl-1,1,2,2-($^2H_4$) isothiocyanate, the internal standard for GC/MS of PEITC. The synthesis is shown below:

Phenylethylamine **PEITC**



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:
We have developed HPLC assays for PEITC, BITC and NITC, as well as performing stability studies on all three compounds. In preliminary animal studies, we have dosed rats with PEITC and obtained blood and urine samples to use in developing our analysis procedures.
Since the major metabolite of PEITC is not commercially available, we synthesized and purified PEITC mercapturic acid conjugate to use as a standard for our analysis of biological samples. The assays for PEITC, PEITC mercapturic acid, the stability of PEITC, and the synthesis of PEITC mercapturic acid were described in the 2000-2001 annual report.

HPLC Assay for BITC
A Waters 1525 Binary Pump and a Waters 2487 Dual λ Absorbance Detector were used. Sample injection was achieved with a Waters 717plus Autosampler, equipped with a Heater/Cooler (25 °C). The column was PartiSphere WVS C18 (5 μm), 4.6 X 125 mm I.D. from Whatman. The column temperature (25 °C) was controlled by Waters 1500 Column Heater. The mobile phase consisted of a mixture of 60% methanol and 40% water eluting at a flow rate of 1.0 ml/min. BITC eluted with a retention time of 7 min. UV detection was optimized to 245 nm.

Stability of BITC. The stability of BITC was determined at various pH values (3, 5, 7.4, 8.4, 9.4, and 10.4) and over time (120 hours). A universal buffer of citrate-phosphate-borate/HCl A universal buffer of citrate-phosphate-borate/HCl (Teorell & Stenhagen) was used in this study. The buffer solutions, with pH of 3.0, 5.0, 7.4, 8.4, 9.4 and 10.4, were spiked with a 0.1mM BITC (in ACN). All samples were left at room temperature and injected at various times throughout a 120-hour period. Injection volume was 50 μl. The stability of BITC is summarized in Table 1. BITC was found to degrade in buffer solutions and the degradation rate followed first order kinetics (Fig. 1). The results showed that BITC is more stable in an acidic environment than in an alkaline one, and is most stable between pH 5 and 7.

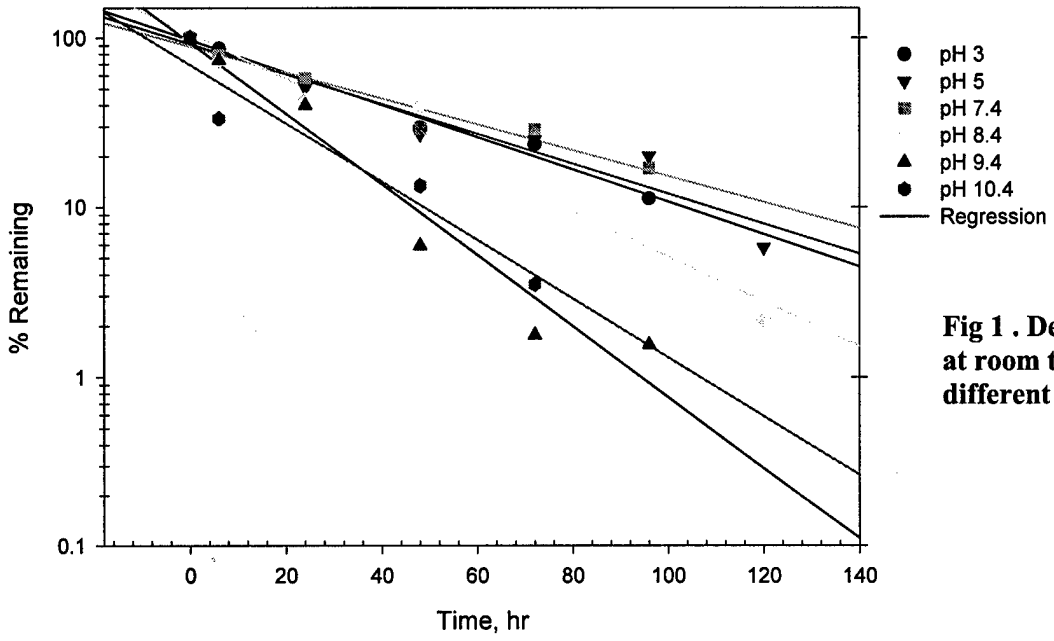


Fig 1 . Degradation of BITC at room temperature at different pH values.

Table 1. Parameters for the first order degradation of BITC in buffer solutions at various pH values.

pH	3	5	7.4	8.4	9.4	10.4
K value ($hours^{-1}$)	0.022	0.020	0.018	0.030	0.048	0.173
Half-life (hours)	31.5	34.0	39.4	22.9	14.4	14.0

HPLC Assay and Stability Studies for NITC

A rapid and sensitive high-performance liquid chromatographic (HPLC) assay for 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 acetonitrile/water (ACN:H_2O 70:30, v/v), and detection by ultraviolet (UV) absorption at 305 nm. 1-NITC and 1-NA were found in rat plasma and urine samples, respectively, with retention time 5.9 and 2.2 min. 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. The standard curve was linear over a concentration range of 10-5000 ng/ml for 1-NITC in plasma and 100-50000 ng/ml for 1-NA in urine ($r > 0.999$). At low (10 ng/ml), medium (500 ng/ml), and high (5000 ng/ml) concentrations of quality control samples (QCs), the within-day and between-day accuracy were 95-106% and 97-103% for 1-NITC in plasma, respectively. The values for within- and between-day precision were 97-100% and 93-97%, respectively. For 1-NA in urine, the within- and between-day accuracy and precision values were 96-106% and 97-99%, respectively. ACN extraction of plasma and urine samples resulted in recoveries of 1-NITC between 93 and 97%, and of 1-NA between 95 and

110%. The stabilities of 1-NITC, 1-NA, and 1-NIC for time course were studied in rat plasma, urine, ACN extracts of plasma and urine, ACN, and universal buffer (citrate-phosphate-borate-HCl, pH 2-12) at room temperature (RT), 4, -20, and -80°C over 96 h. 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 at RT, 1-NITC degraded rapidly at pH values ranging from 2 to 12. 1-NA was stable in all tested matrix at all temperatures. 1-NIC was unstable in plasma, urine, and ACN extracts of 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. The validated HPLC assay was used in a pharmacokinetic study of 1-NITC following intravenous (i.v.) bolus administration of 25 mg/kg dose to a female Spraque-Dawley rat. 1-NITC and 1-NA were detected and quantified in rat plasma and urine. Based on noncompartmental analysis, values for 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.
(Manuscript to be submitted is attached in Appendix D)

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

Overview:
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 in the present study.

HPLC Assay for Doxorubicin
The Waters HPLC system (Milford, MA) consisted of a 1525 binary pump, a 717plus autosampler, a Model 5HC column oven, a 1525 fluorescence detector, and a 2487 UV detector. The column and autosampler temperatures were at room temperature. The reversed-phased chromatography was performed with a stain-steel less Whatmann (Clifton, NJ) Partisphere C-18 5 μm column 125 × 4.6 mm protected by a μBondapak-CN precolumn insert, and eluted isocratically with a mobile phase consisted of H_2O:ACN (30:70, v/v). The flow rate was set at 1.0 ml/ml and the injection volume was 10 μl. The eluent was monitored fluorimetrically at an excitation wavelength of 480 nm and an emission wavelength of 560 nm. The detection and integration of chromatographic peaks was performed by the Breeze data analysis system.

The LLQ of DOX by HPLC with fluorescence detection was identified as 10 ng (1 μg/ml with 10 μl injection) since the height of sample peak is 10-fold greater than the noise level. The retention time (R_t) for DOX was 5.44 min. The other peak with a R_t around 7 min was identified as some interference(s) in the solvent ACN. (Fig. 2) Our data are consistent with previously reported LLQ data, which range from 2.5 to 20 ng.
Based on the result of LLQ, the linearity of standard curve was tested from 1.0 to 10.0 μg/ml with the same injection (i.e. 10 μl). The correlation coefficient *r* was greater than 0.999.

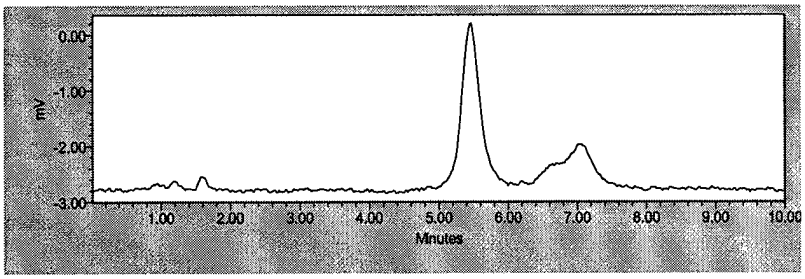


Fig. 2. HPLC assay of doxorubicin (10 μl of a 5 μg/ml solution was injected)

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: Studies have not yet been started to address this task. We will begin studies to evaluate the effects of the ITCs on DOX kinetics (Task 6), plus the metabolism and biliary excretion of DOX (Task 7) this summer. Our progress has been somewhat slow in this area due to problems in developing extraction methods, and assays with sufficient sensitivity to analyze plasma concentrations of unchanged drug (especially PEITC), as well as problems with limited stability of the compounds (especially NITC).

Pharmacokinetics of α-naphthylisothiocyanate (NITC) in rats. To date we have analyzed the pharmacokinetics of NITC in rats. α-naphthylisothiocyanate (1-NITC) and its metabolite α-naphthylamine (1-NA) were determined in plasma and urine samples of three female Spraque-Dawley rats after an i.v. bolus of 25 mg/kg dose of 1-NITC. 1-NITC and 1-NA were detected and quantified in rat plasma and urine samples, respectively using our HPLC method. Based on noncompartmental analysis by WinNonLin 2.1, the fitted parameters clearance (CL = 2.29 ± 0.81 l/kg/h), volume of distribution (V = 16.8 ± 3.55 l/kg), and half life ($t_{1/2}$ = 5.26 ± 0.77 h) were determined for 1-NITC.

Table 2. Urinary recovery of α-naphthylamine (1-NA) following administration of 1-NITC to rats.

	Rat 1	Rat 2	Rat 3
Dose of 1-NITC (mg)	6.67	6.17	6.35
Urinary recovery of 1-NA (mg)	0.108	0.135	0.119
Percent recovery of 1-NA	2.09	2.82	2.46

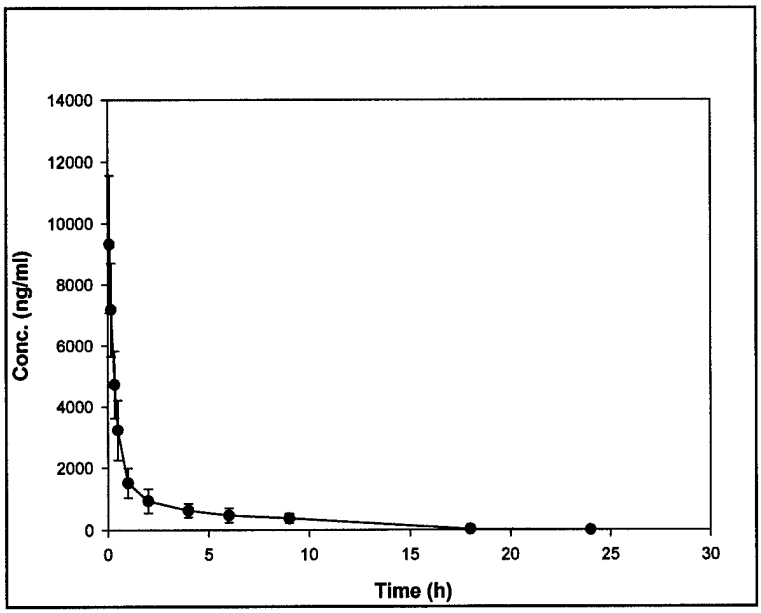


Fig. 3. Plasma concentration vs. time profile for 1-NITC following the i.v. administration of 25 mg/kg to rats. Data are presented as mean ± SD, n=3.

Tasks for Year 3

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)

These studies are on track to be started in the Summer of 2002.

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)

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

We are on track to complete these studies in the latter part of Year 3.

KEY RESEARCH ACCOMPLISHMENTS

- Organic isothiocyanates, also known as mustard oils, are present in cruciferous vegetables in mg quantities. We identified three organic isothiocyanates (naphthyl isothiocyanate (NITC), phenethyl isothiocyanate (PEITC), benzyl isothiocyanate (BITC)) that significantly increase the cellular accumulation of daunomycin (DNM) and vinblastine (VBL) due to inhibition of P-glycoprotein-mediated efflux.

- BITC and PEITC inhibited MCF-7/adr cell growth with IC_{50}'s of 10.37 ± 0.31μM and 13.98 ± 0.54 μM for a 2-hour exposure and 5.95 ± 0.10μM and 7.32 ± 0.25 μM for a 48-hour exposure (mean ± SE). NITC exhibited no cytotoxicity at similar concentrations. Cytotoxicity was also determined on a normal mammary cell line, MCF-12A. The IC_{50} values for BITC, PEITC and NITC were 8.07 ± 0.29 μM, 7.71 ± 0.074 μM and 33.63 ± 1.69 μM, respectively, after a 48-hour exposure. There is very limited data in the literature regarding the effects of ITCs on cell growth and this information is important in the evaluation of these drugs as chemosensitizing or chemopreventive agents.

- Reversed phase HPLC assays for NITC and BITC were developed and specificity and sensitivity determined.

- Stability studies indicated that BITC is most stable at neutral or acid pH values with a half-life in pH 7.4 buffer of 39.4 hours at RT. On the other hand, NITC is very unstable in buffer, plasma and urine. Although, NITC is stable in acetonitrile at all temperatures, it degrades rapidly at RT and 4°C in buffer, plasma and urine samples.

- The pharmacokinetics and metabolism of 1-NITC were characterized in rats after a 25 mg/kg dose. The values for clearance, volume of distribution and half-life were 2.29 ± 0.81 L/kg/h, 16.8 ± 3.55 L/kg, and 5.26 ± 0.77 hours, respectively. Only about 2.5% of the dose was recovered in the urine as the metabolite, α-naphthylamine This study represents the first determination of the pharmacokinetics of α-naphthylisothiocyanate.

REPORTABLE OUTCOMES

Abstracts (in Appendix E):

1. Cytotoxicity of Dietary Organic Isothiocyanates in Human Breast Cancer Cells. E. Tseng and M. E. Morris. Poster presentation to the American Association of Pharmaceutical Scientists Annual Meeting, October 2001. Published in Pharmaceutical Research Suppl, 2001.
2. 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, Submitted to the American Association of Pharmaceutical Scientists Annual Meeting, November 2002.

3. HPLC Assay and Stability of Phenethyl Isothiocyanate, Y. Kuo, Y. Ji, L. Predko and M.E. Morris, Submitted to the American Association of Pharmaceutical Scientists Annual Meeting, November 2002.
4. 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.

Manuscripts:

1. E. Tseng, A. Kamath and M.E. Morris. Effect of organic isothiocyanates on P-glycoprotein- and MRP1-mediated transport of Daunomycin and Vinblastine, Pharmaceutical Research, accepted for publication.

Graduate Students Participating in this Research as a part of their educational program::

Elaine Tseng, B.S/M.S candidate (graduated Sept. 2001)
Yushin Kuo, M.S. Candidate (degree expected, Sept, 2002)
Yan Ji, Ph.D. candidate (degree expected, 2005)

Undergraduate Students Participating in this Research:

Bo Lin (Pharm.D. degree expected May, 2004)
Lisa Predko (Pharm.D. degree conferred May 2002)

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, as well as indirect effects mediated by the down-regulation of P-gp, on multidrug resistance in breast cancer cells.
2. PEITC, BITC and NITC exhibit cytotoxicity in human breast cancer and normal mammary cells. This inhibition of cellular growth may be important for the chemopreventive or chemotherapeutic properties of these compounds.
3. HPLC assays were developed to determine PEITC, BITC and NITC concentrations in biological fluids. A GC/MS method for PEITC is under development to increase the sensitivity of our analytical methods.
4. ^{14}C-PEITC was synthesized for use in transport, metabolism and pharmacokinetic studies.
5. The pharmacokinetics and metabolism of NITC were determined in rats following a 25 mg/kg i.v. dose. The values for clearance, volume of distribution and half-life were 2.29 ± 0.81 L/kg/h, 16.8 ± 3.55 L/kg, and 5.26 ± 0.77 hours, respectively. This represents the first determination of the pharmacokinetics of α-naphthylisothiocyanate.

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Appendices

Appendix A – Accepted Publication - E. Tseng, A. Kamath and M.E. Morris. Effect of organic isothiocyanates on P-glycoprotein- and MRP1-mediated transport of Daunomycin and Vinblastine, Pharmaceutical Research

Appendix B – Manuscript to be submitted – E. Tseng, E.A. Raybon and M.E. Morris. Cytotoxicity of organic isothiocyanates in the human cell lines, MCF-7 and MCF-12A.

Appendix C – Protocol for ^{14}C-PEITC Synthesis

Appendix D – Manuscript to be submitted – Determination of α-naphthylisothiocyanate and metabolites α-naphthylamine and α-naphthylisocyanate in rat plasma and urine by high-performance liquid chromatography.

Appendix E - Abstracts

1. Cytotoxicity of Dietary Organic Isothiocyanates in Human Breast Cancer Cells. E. Tseng and M. E. Morris. Poster presentation to the American Association of Pharmaceutical Scientists Annual Meeting, October 2001. Published in Pharmaceutical Research Suppl, 2001.
2. 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, Submitted to the American Association of Pharmaceutical Scientists Annual Meeting, November 2002.
3. HPLC Assay and Stability of Phenethyl Isothiocyanate, Y. Kuo, Y. Ji, L. Predko and M.E. Morris, Submitted to the American Association of Pharmaceutical Scientists Annual Meeting, November 2002.
4. 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.

Effect of Organic Isothiocyanates on the P-glycoprotein- and MRP1-mediated Transport of Daunomycin and Vinblastine

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Running title: Organic Isothiocyanates and MDR

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Abbreviations: AITC allylisothiocyanate; BITC benzylisothiocyanate; DNM daunomycin; HITC hexylisothiocyanate; ERY erysolin; ITC isothiocyanate; MDR multidrug resistance; MRP1 multidrug resistance-associated protein; NITC 1-naphthylisothiocyanate; PBITC phenylbutylisothiocyanate; PEITC phenethylisothiocyanate; P-gp P-glycoprotein; PHITC phenylhexylisothiocyanate; PITC phenylisothiocyanate; PPITC phenylpropylisothiocyanate; VBL vinblastine.

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Abstract

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 (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.

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.

Introduction

What may be considered a major set back 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 following 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 physiological 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 190kD 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 ATP-binding cassette (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, while 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. verpamail, 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 due to dose-related toxicities that occur at the doses necessary to achieve MDR reversal [8].

The main objective of the present study is to examine the effects of dietary organic isothiocyanates (ITCs) on P-gp- 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 mg 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) following 2-hour 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 while PANC-1 cells overexpress MRP1.

Materials:

Erysolin, Phenyl ITC, β-phenylethyl ITC, α-naphthyl ITC, and verapamil were obtained from Sigma Chemical Co. (St. Louis, MO). Benzyl ITC, n-hexyl ITC, and allyl ITC were obtained from Aldrich (St. Louis, MO). Sulforaphane and erucin were purchased from ICN (Aurora, OH) and phenylpropyl ITC and phenybutyl ITC were purchased from LKT Laboratories (St. Paul, MN). Phenylhexyl ITC was a gift from National Cancer Institute-Chemopreventive Division (Bethesda MD). Radiolabelled [^{3}H]-daunomycin (14.4 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA) and [^{3}H]-vinblastine Sulfate (7.3 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Cell culture reagents were supplied by GIBCO BRL (Buffalo, NY) and cell culture flasks and dishes were purchased from Falcon (Becton Dickinson, Franklin Lakes, NJ). Biodegradable liquid scintillation cocktail was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Commassie blue dye reagent was obtained from Bio-Rad laboratory (Hercules, CA). 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). The monoclonal antibodies C219 and MRPr1 were obtained from Kamiya Biomedical Co. (Seattle WA).

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 kDa), phosphorylase b (97.4 kDa), and ovalbumin (46 kDa). 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, Hercules, CA)

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) BSA, incubated with C219 (1μg/ml) or MRPr1 (1:30) antibodies in blocking buffer for 2 hours at room temperature. The filters were then washed in washing buffer (20mM Tris base, 137mM 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, C.A.) (for MRPr1), in blocking buffer for 2 hours. 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, 2mM 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-0.53mM EDTA. Cells were grown in 75mm^2 plastic culture flasks, seeded in 35mm^2 plastic culture dishes for accumulation studies. Experiments were performed 2-3 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 2-3 days with 0.25% trypsin- 2.6mM EDTA. For experiments, cells were seeded on 35mm^2 dishes at a density of 10^6 cells per dish and used two 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 $CaCl_2$, 1.2mM $MgCl_2 6H_20$, 10mM HEPES, pH 7.4). One ml of incubation buffer containing 0.05μM of [^{3}H]-DNM or 0.05μM [^{3}H]-VBL and 100μM of ITC was added to the dish and incubated for two hours. 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 iced-cold stop solution (137 mM NaCl, 14mM Tris-base, pH 7.4). One ml of 0.5% Triton-X-100 or 0.3N 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, Hercules, CA) 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 are 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 (figure 1). The results found in this experiment confirmed those in the literature [13,14].

Time course study. The time course of uptake of 0.05μM ^{3}H-DNM in the presence and absence of 100μM verapamil, a typical inhibitor, was examined in sensitive (MCF-7/WT) and resistant (MCF-7/ADR) cells for up to two hours (figure 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 hours in both the sensitive and resistant MCF-7 cells.

Daunomycin Accumulation. The effect of various organic isothiocyanates on DNM accumulation was examined in MCF-7/WT cells (figure 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 NITC which increased DNM accumulation by 4-fold; 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).

Vinblastine Accumulation. The uptake of VBL was examined in the presence and absence of ITCs. In MCF-7/ADR cells, verapamil significantly increased the accumulation of VBL by 33-fold, phenylhexyl ITC by 10-fold and NITC by 40-fold (figure 4). The greatest effects of the ITCs on accumulation were seen for VBL in MCF-7/ADR cells.

PANC-1 Cells

Daunomycin Accumulation. In PANC-1 cells, PEITC, erysolin, NITC and verapamil were able to significantly increase DNM accumulation (figure 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).

Vinblastine 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 (figure 6). The correlation between ITC inhibition (% control values) for DNM and VBL in PANC-1 cells had an r^2 value of 0.37 ($p<0.05$). (Results 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 ($p<0.05$)(figure 7A) and 0.86 for VBL ($p<0.005$)(figure 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 due to 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 histological 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 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 isothiocyanates 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 due to the direct inhibition

and/or down regulation of the CYP-450s responsible for carcinogen activation [16]. In addition, isothiocyanates 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-hour 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. Since 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 while vinblastine, actinomycin D and etoposide inhibit transport of both dyes. This suggests that

compounds like DNM may represent a substrate for only one site, while 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-oestradiol 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, since 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, following a 2-hour accumulation study; the compounds were ineffective at a concentration of 10 μM. However, concentration-dependent effects after prolonged exposures have not been examined. Following 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 due to 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, while 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 isothiocyanate 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. 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 [30]. 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 six-fold higher plasma level of paclitaxel in the latter, at least partly due to increased bioavailability [31]. We found that 100 μM concentrations of NITC,

BITC and PEITC could significantly increase the 2-hour 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 due to 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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Figure Legends

Figure 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 under Methods).

Figure 2. Time course of DNM uptake in MCF-7 sensitive and resistant cells.

DNM (0.05μM) uptake was measured in the presence and absence of verapamil.

( ) MCF-7/ADR + Verapamil (100μM), ( ) MCF-7/ADR control, ( ) MCF-7/WT + Verapamil (100uM), ( ) MCF-7/WT control. Data is mean ± SD of data from one representative study. The study was repeated with similar results.

Figure 3. Effect of Organic ITCs on DNM Accumulation in MCF-7 cells.

The two-hour accumulation of 0.05μM DNM was measured in the presence of various ITCs (100μM). Control represents the uptake in the absence of ITCs. Each bar represents mean±SE. N=9-12, *p<0.001.

Figure 4. Effect of Organic ITCs on VBL Accumulation in MCF-7/ADR cells.

The two-hour accumulation of 0.05μM VBL was measured in the presence of various ITCs (100μM). Control represents the uptake in the absence of ITCs. Each bar represents mean±SE. N=9-12, *p<0.001.

Figure 5. Effect of Organic ITCs on DNM Accumulation in PANC-1 cells.

The two-hour accumulation of 0.05μM DNM was measured in the presence of various ITCs (100uM). Control represents the uptake in the absence of ITCs. Each bar represents mean±SE. N=9-12, *p<0.001.

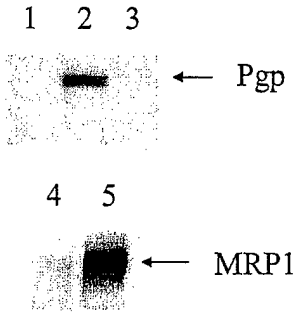
Figure 6. Effect of Organic ITCs on VBL Accumulation in PANC-1 cells.

The two-hour accumulation of 0.05μM VBL was measured in the presence of various ITCs (100μM). Control represents the uptake in the absence of ITCs. Each bar represents mean±SE. N=9-12, *p<0.001.

Figure 7. Correlation between ITC inhibition in MCF-7/ADR cells and PANC-1 cells.

(A) The relationship between ITC inhibition of DNM 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 VBL in MCF-7/ADR cells with that in PANC-1 cells. $R^2 = 0.86$, $p<0.005$.

Figure 1



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

Figure 2

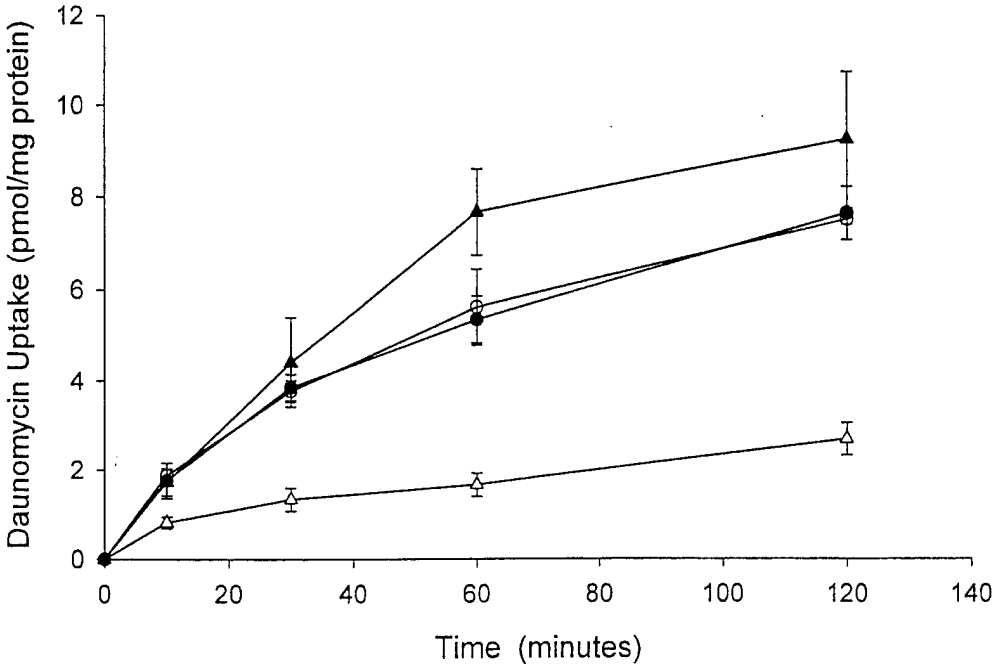

Daunomycin Uptake (pmol/mg protein)
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
Time (minutes)

Figure 3

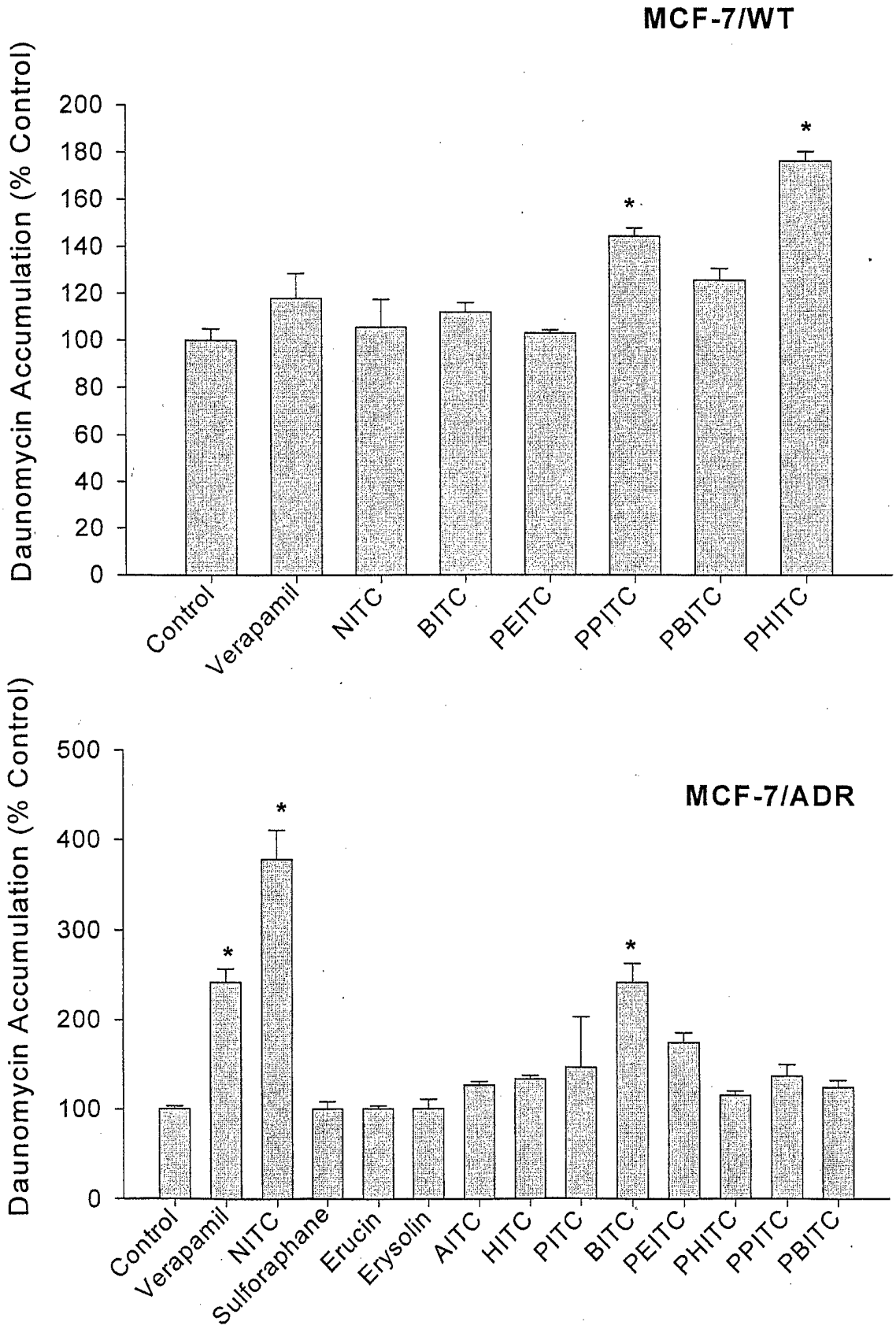

MCF-7/WT
Daunomycin Accumulation (% Control)
0
20
40
60
80
100
120
140
160
180
200
Control
Verapamil
NITC
BITC
PEITC
PPITC
PBITC
PHITC
*
*
MCF-7/ADR
Daunomycin Accumulation (% Control)
0
100
200
300
400
500
Control
Verapamil
NITC
Sulforaphane
Erucin
Erysolin
AITC
HITC
PITC
BITC
PEITC
PHITC
PPITC
PBITC
*
*
*

Figure 4

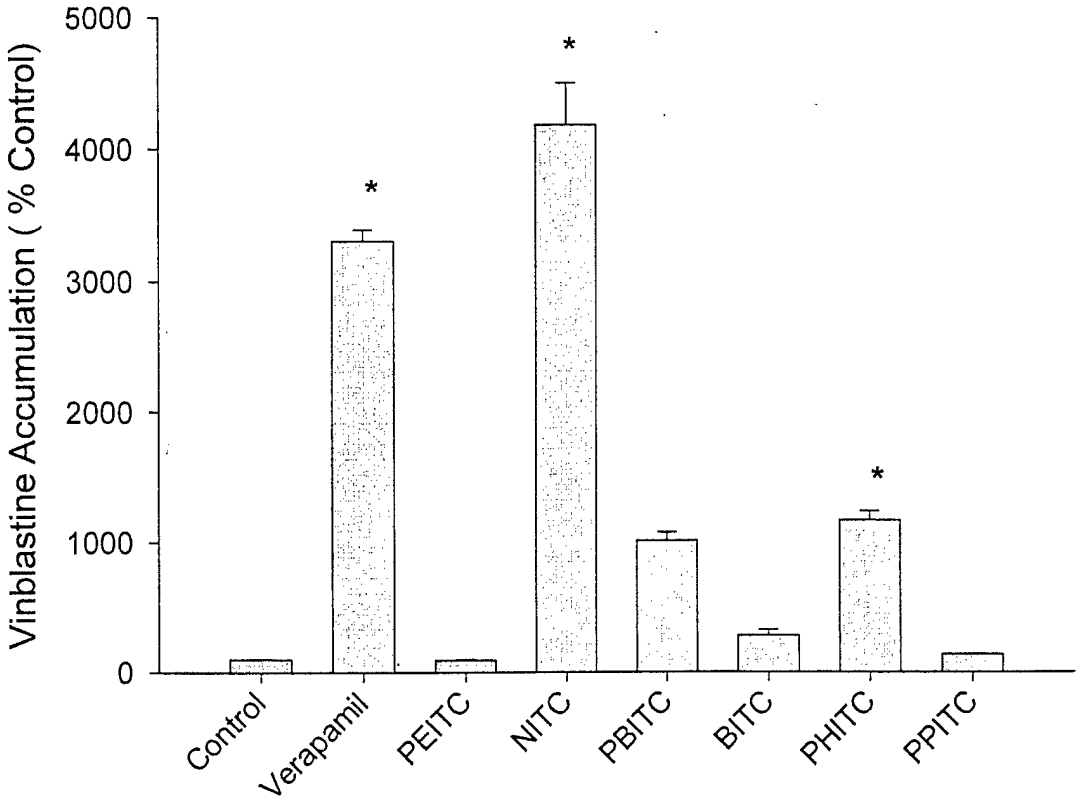

Vinblastine Accumulation (% Control)
5000
4000
3000
2000
1000
0
*
*
*
Control
Verapamil
PEITC
NITC
PBITC
BITC
PHITC
PPITC

Figure 5

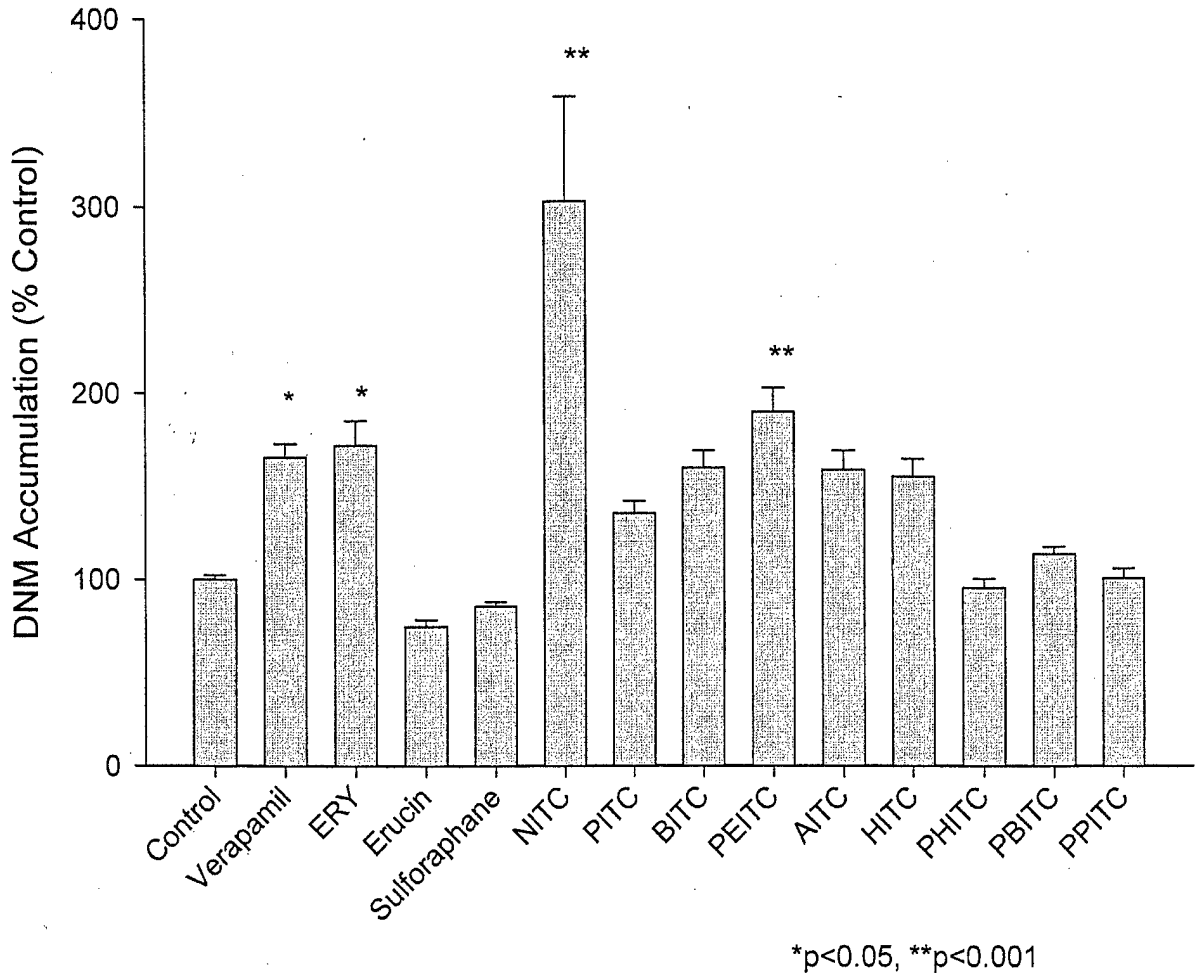

DNM Accumulation (% Control)
400
300
200
100
0
*
*
**
**
Control
Verapamil
ERY
Erucin
Sulforaphane
NITC
PITC
BITC
PEITC
AITC
HITC
PHITC
PBITC
PPITC
*p<0.05, **p<0.001

Figure 6

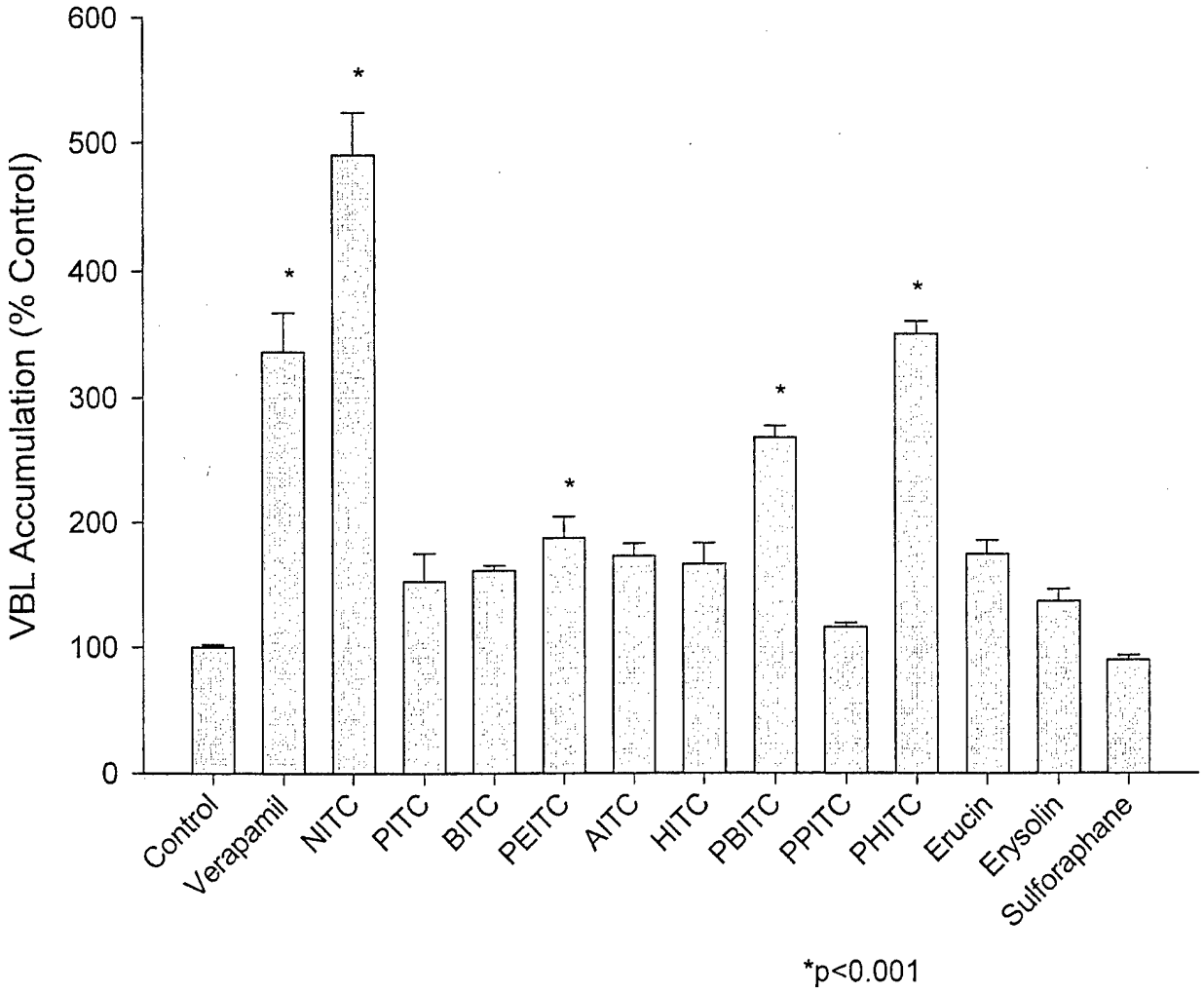
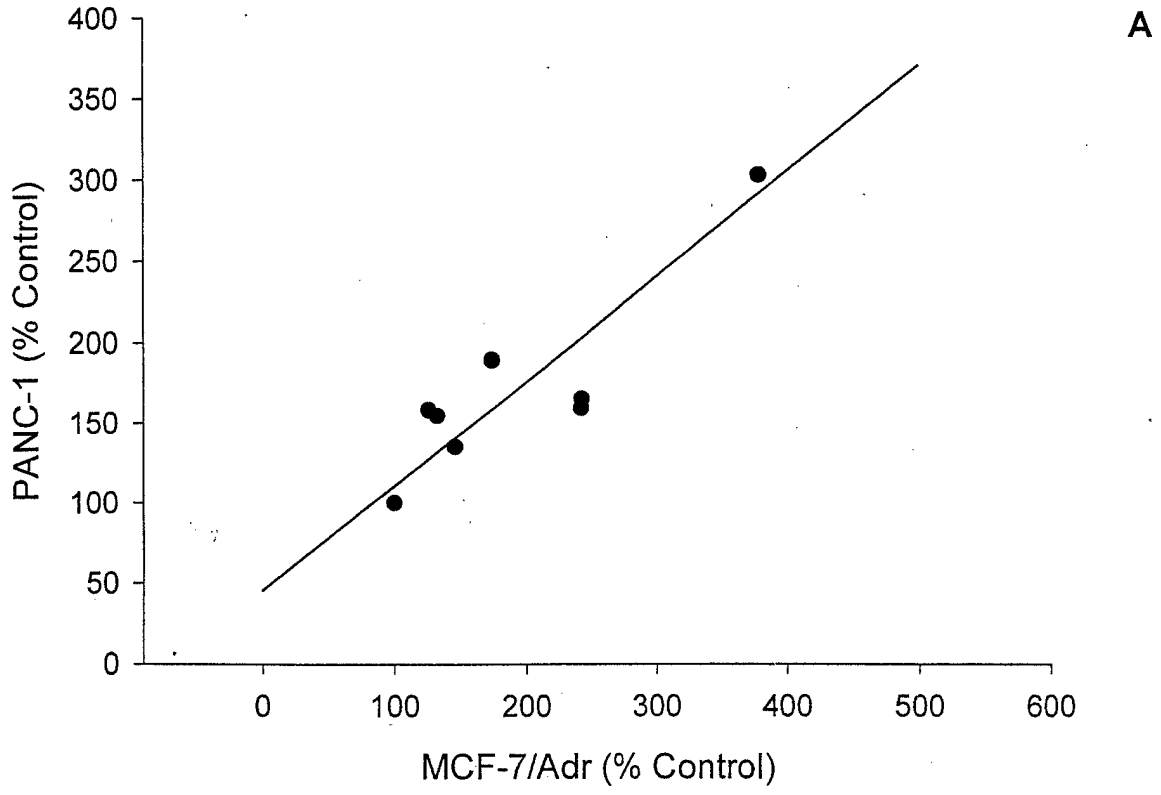
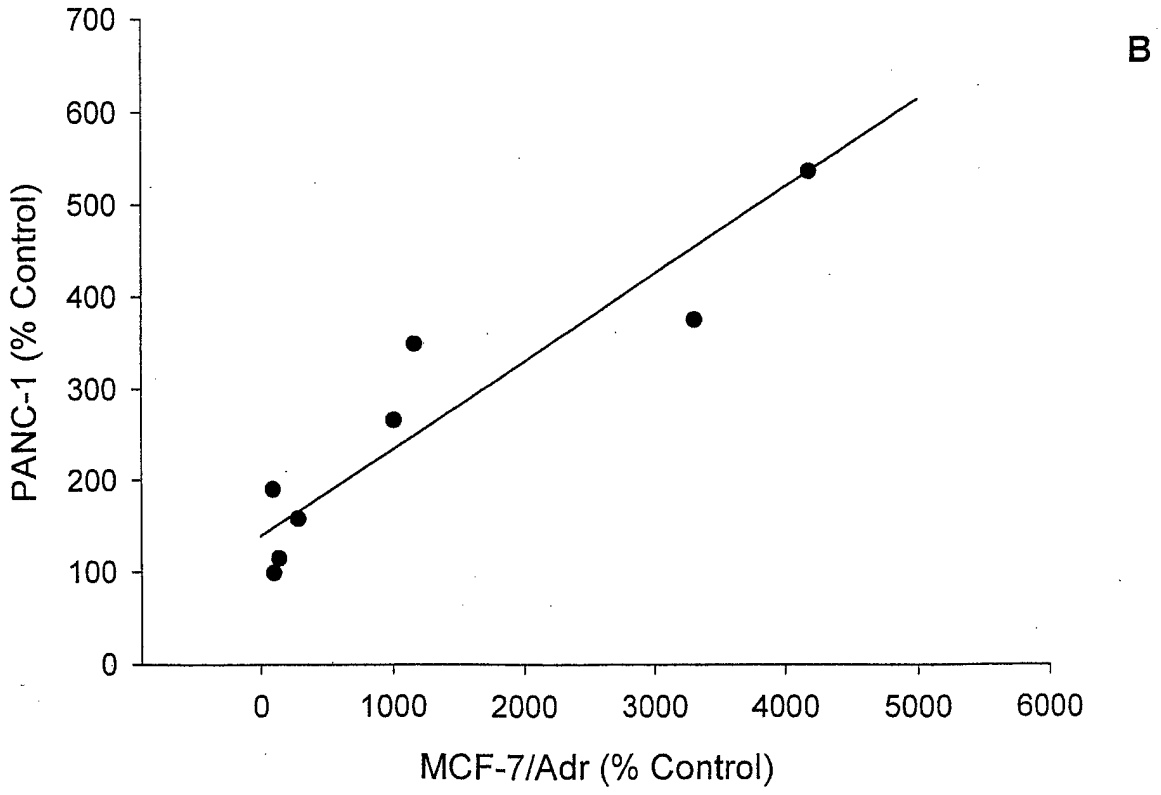

VBL Accumulation (% Control)
600
500
400
300
200
100
0
Control
Verapamil
NITC
PITC
BITC
PEITC
AITC
HITC
PBITC
PPITC
PHITC
Erucin
Erysolin
Sulforaphane
*p<0.001

Figure 7 A and B





Cytotoxicity of Organic Isothiocyanates in the Human Cell Lines, MCF-7 and MCF-12A

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Abstract

Purpose. Organic isothiocyanates (ITCs) (mustard oils) are present in cruciferous vegetables and are known to have cancer chemopreventative properties due to their inhibition of carcinogen metabolic activation. The purpose of this investigation was to examine the cytotoxicity of 1-naphthyl isothiocyanate (NITC), benzyl isothiocyanate (BITC) and β-phenylethyl isothiocyanate (PEITC) in a human breast cancer and human mammary epithelium cell line, MCF-7 and MCF-12A respectively. **Methods.** The cytotoxicity of PEITC, BITC, NITC, in concentrations varying from 0.1μM to 50μM and daunomycin (DNM) and vinblastine (VBL) were examined following various exposure times, up to 48 hours, in the cultured cells. Cell growth was determined by a sulforhodamine B assay. **Results.** The IC_{50} of DNM and VBL in MCF-7/Adr cells were 40.10 ± 1.74 μM and 9.99 ± 0.803 μM, respectively, following a 2-hour exposure and decreased to 7.12 ± 0.42 μM and 0.106 ± 0.004 μM following a 48-hour exposure. The corresponding values for BITC and PEITC are 10.37 ± 0.31μM and 13.98 ± 0.54 μM for a 2-hour exposure and 5.95 ± 0.10μM and 7.32 ± 0.25 μM for a 48-hour exposure. IC_{50} values for NITC in MCF-7/Adr cells could not be determined since there was limited toxicity at concentrations up to 50 μM. In MCF-12A cells, the IC_{50} of DNM was not able to be determined due to the limited toxicity seen at concentrations up to 100μM at the 48-hour endpoint. Corresponding values for BITC, PEITC, and NITC were 8.07 ± 0.29 μM, 7.71 ± 0.074 μM and 33.63 ± 1.69 μM respectively. The differences in IC_{50} between cancer and normal cells may be due to a number of factors in these two cell lines, including intrinsic differences in sensitivity to the cytotoxicity of these compounds, differences in free drug concentrations due to protein binding considerations due to differences in the protein content of media, differences in the metabolism of these compounds, or

differences in the intracellular accumulation of these compounds. **Conclusions.** BITC and PEITC, which are present in cruciferous vegetables in mg quantities, can inhibit the growth of human breast cancer cells as well as normal human mammary epithelium cells, at concentrations similar to the chemotherapeutic drug daunorubicin. This effect on cell growth may contribute to the cancer chemopreventive properties of ITCs by suppression of the growth of preclinical tumors, and/or may indicate a potential use of these compounds as chemotherapeutic agents in cancer treatment.

Introduction

Organic isothiocyanates (ITCs) are plant derived dietary compounds commonly known as mustard oils. Present in the Brassica genus of the Cruciferae family, they are found in vegetables such as cabbage, broccoli, brussels sprouts, and cauliflower, in the form of glucosinolates, the biosynthetic precursors of isothiocyanates in plants. When vegetables are chewed, the glucosinolates are cleaved by the enzyme myrosinase (1) to form isothiocyanates. Glucosinolate levels have been estimated to be as high as 180mg/g in some vegetables (2). Over 20 natural and synthetic ITCs have demonstrated cancer preventive properties in animals treated with chemical carcinogens, including polycyclic aromatic hydrocarbons and nitrosamines (3-6). There is substantial evidence that the inhibition of tumorigenesis is partly due to the direct inhibition and/or downregulation of the CYP-450s responsible for carcinogen activation, resulting in decreased amounts of ultimate carcinogens formed (7). In addition, ITCs have also demonstrated the ability to induce certain phase II enzymes responsible for the detoxification of electrophilic intermediates formed during phase I metabolism (7). For example, ITCs can inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (NNK)-induced carcinogenesis by inhibiting the microsomal metabolism of NNK to reactive species that form methyl and pyridyloxobutyl adducts in DNA (8). ITCs may also be able to suppress tumor cell growth. Recent studies have indicated that certain ITCs can affect the cell cycle: sulforaphane induces cell cycle arrest and apoptosis in HT29 human colon cancer cells (9); PEITC also induces apoptosis in vitro (10); and AITC can induce apoptosis in dimethylhydrazine (DMH)-induced rat colon cancer (11).

The main objective of this investigation was to examine the cytotoxic effects of benzyl isothiocyanate (BITC), β-phenethyl isothiocyanate (PEITC), and α-naphthyl isothiocyanate

(NITC) in a human breast cancer cell line (MCF-7/ADR) and a human mammary epithelium cell line (MCF-12A). Results were compared with the cytotoxicity observed for two chemotherapeutic agents, daunomycin (DNM) and vinblastine (VBL).

Materials

β-phenylethyl ITC, α-naphthyl ITC, daunorubicin HCl and vinblastine sulfate were obtained from Sigma Chemical Co. (St. Louis, MO). Benzyl ITC was obtained from Aldrich (St. Louis, MO). Cell culture reagents were supplied by GIBCO BRL (Buffalo, NY). Cell culture flasks and 96-well plates were purchased from Falcon (Becton Dickinson, Franklin Lakes, NJ). The MCF-7/ADR cell line was a gift from Dr. Ralph Bernacki (Roswell Park Cancer Institute). MCF-12A cell line was purchased from ATCC. Epidermal growth factor 20ng/ml was obtained from BD Biosciences (Bedford, MA). Cholera toxin 100ng/ml, Insulin 10μg/ml and Hydrocortisone 500ng/ml were purchased from Sigma Chemical Co.(St. Louis, MO).

Cell Culture

MCF-7/ADR cells used between passages 16-24, were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2mM 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-0.53mM EDTA. Cells were grown in 75mm^2 plastic culture flasks and seeded in 96 well plates. Experiments were performed 48 hours days after seeding.

MCF-12A cells, used between passages 56-58, were grown in a 1:1 mixture of DME media and HAM's F12 media supplemented with 20 ng/ml epidermal growth factor, 100ng/ml cholera toxin, 10μg/ml insulin and 500 ng/ml hydrocortisone, 95%: horse serum 5%. Cells were incubated at 37°C supplemented with 5% CO_2/95% air. Cells were subcultured using 0.25% trypsin-0.03% EDTA in PBS. Cells were grown in 75mm^2 plastic culture flasks and seeded in 96 well plates. Experiments were performed 48 hours days after seeding.

Cytotoxicity Assay

Cells were plated at a density of 5,000 cells/well for 48 hours prior to drug exposure. The doubling rate for MCF-7/ADR cells is ~28 hours, therefore plating cells for 48 hours makes certain that cells are in their exponential phase when exposed to drugs. Cells are then exposed to 200μL of growth media in the absence or presence of increasing concentrations of NITC, BITC, PEITC (0-50μM), DNM and VBL (0-500μM) at time intervals up to 48 hours. At the end of the incubation period,, the cells were rinsed twice with 1X PBS and fresh media was added. Control wells were incubated with the vehicle DMSO; the percentage of DMSO in media was kept constant (0.5 %) in all studies. At 48 hours, media was removed and a sulforhodamine B (SRB) assay was performed (12). Briefly, cells were fixed to the plate with 100μL of 10% trichloroacetic acid and stored at 4°C for one hour. Cells were then rinsed 5 times with water and allowed to dry for 5-10 minutes. A 100μL-aliquot of SRB 0.4% dye was added to each well for 15 minutes and the wells were rinsed 3-4 times with 1% acetic acid. Plates were allowed to air dry for another 5-10 minutes then solubilzed with 10mM Tris-base for 5 minutes. Absorbance was read at 570nM with a Spectra Rainbow plate reader (Tecan US, SLT Lab Instruments, Research Triangle Park, NC).

Data Analysis

Data is fitted to a sigmoidal E_{max} equation using Win Nonlin (Pharsight Corp., Mountain View, CA.).

$$E = E_0 - \left(\frac{E_{\max} \times C^{\gamma}}{IC_{50}^{\gamma} + C^{\gamma}} \right) \qquad \text{(Equation 1)}$$

Statistical analysis was determined by a one-way ANOVA followed by Dunnett's post hoc test using the analysis software GraphPad Prism (GraphPad Inc., San Diego CA.) Differences were considered to be significant when $p<0.05$.

Results

Cytotoxicity as a function of time exposure. The general pattern seen in the MCF-7/ADR cell line is a decrease in IC_{50} values with longer exposure times to the test drugs (Figure 10). The IC_{50}'s for DNM and VBL significantly decreased over the 48-hour exposure period, with the lowest values obtained following a 48-hour exposure. The IC_{50}'s for the ITCs were significantly smaller following a 6-hour exposure compared with a 2-hour exposure, but were unchanged following 6- and 48-hour exposure times.

Cytotoxicity Studies with MCF-7/ADR cells. The IC_{50} of DNM in this cell line determined following a 48-hour exposure was 7.12 ± 0.42 μM, comparable to that reported in the literature (NCI/NIH, 2001). The IC_{50} of vinblastine observed in this cell line was 0.106 ± 0.004 μM that is also comparable to literature reports (NCI/NIH, 2001). In the resistant cancer cell line, the IC_{50} values for the ITCs and DNM were generally lower than the MCF-12A cells. The general potency, determined after a 48-hour exposure, was VBL>BITC>PEITC>DNM>NITC. (Table 1, Figures 1-4). An IC_{50} value for NITC could not be determined because there was only approximately 80% inhibition at the highest concentration (50 μM) used in this study. PEITC and BITC were more cytotoxic than DNM after an exposure of 6 hours and comparable after a 48-hour exposure.

Cytotoxicity Studies with MCF-12A cells. The IC_{50} of DNM was not able to be determined at concentration up to 100μM at a 48-hour exposure time in the MCF-12A cells. When BITC, PEITC, and NITC were incubated with MCF-12A cells in vitro, there was a dose-dependant inhibition of cell growth (Table 2, Figures 5-9). BITC and PEITC tested with a concentration range of 0-100μM inhibited the growth of MCF-12A cells with similar potency: IC_{50} values

ranged from 7.71-8.07 μM for a 48-hour exposure. However, NITC exhibited less toxicity than BITC and PEITC. The IC_{50} values for NITC averaged 33.62 μM for a 48-hour exposure.

Discussion

The capacity for organic isothiocyanates to block chemical carcinogenesis was first recognized over 30 years ago with NITC. ITCs have been demonstrated to be potent chemopreventive agents in numerous animal models for cancer (5, 13-15). One mechanism involved in this action is the inhibition of carcinogen metabolic activation via inhibition of cytochrome P-450 enzymes; increased detoxification of carcinogens through induction of glutathione and glucuronyl transferases or quinone reductase may also be important (16). Specifically, it has been demonstrated that BITC can inhibit CYP 1A1, 2B1, and 2E1 (17), while PEITC inactivates CYP 2E1(18). ITCs are able to increase glutathione-S-transferase and quinone reductase levels in various rat organs by 2-4 fold (7).

In this investigation we found that BITC and PEITC inhibit cell growth in breast cancer cells at similar concentrations as observed for DNM. These concentrations are 4-6 fold lower than the IC_{50} for the isoflavonoid, genistein, a compound that has also been studied in MCF-7 cells. Genistein had the lowest IC_{50} for the flavonoids tested in MCF-7 cells (19). Although the plasma concentrations of ITCs following dietary consumption in humans is unknown, it has been estimated that the consumption of 100 g of broccoli could release 40 μmoles of the ITC, sulforaphane, which would result in low μM concentrations in plasma (20). PEITC is a naturally occurring ITC, being found as its glucosinolate conjugate gluconasturiin in vegetables including watercress. Consumption of about 30 g of watercress releases about 46.5 μmoles of PEITC (21). Another factor, which will highly influence plasma ITC levels, is the polymorphic expression of the major metabolizing enzyme, glutathione-S-transferase M1 (GSTM1). GSTM1 is polymorphic in that 50% of the white population in Europe and North America has no GSTM1. Recent clinical studies have suggested that subjects that lack GSTM1 may have a greater cancer

protective effect following the ingestion of cruciferous vegetables. Subjects with a deficiency of GSTM1 who consume broccoli have greater protection against colon cancer (22). In a study of Chinese subjects, among all subjects with detectable levels of ITCs in their urine, there was a 40% decreased risk of lung cancer. In those subjects with detectable levels of ITCs in their urine and a GSTM1 deficiency, there was a 64% decrease in the risk of developing lung cancer (23).

The mechanism underlying the cytotoxicity of ITCs in unknown, but apoptosis may play an important role. PEITC is capable of inducing apoptosis in a dose-dependant manner in HeLa cells, stimulating proteolytic activity of caspase-3-like proteases, leading to the cleavage of PARP (poly-(ADP-ribose)polymerase), usually occurring within hours (24). Experiments carried out in p53 +/+ and p53 -/- cell lines clearly demonstrate that apoptosis was produced by PEITC in p53 +/+ cells and not in the p53 -/-, suggesting that increased p53 protein and p53-dependent transcriptional activity may be associated with PEITC-induced apoptosis and antitumor activity (10). Lastly, PEITC can induce c-Jun N-terminal kinase 1 (JNK1); stress-activated protein kinases involved in apoptosis. Activation of the JNK pathway is required for apoptosis induced by growth factor withdrawal (25) ceramide (26) UV-C and γ-radiation and is upstream of caspase activation in isothiocyanate-induced caspase activity and apoptosis (27). While there are no reported cellular toxicities of NITC, there have been reported cases of hepatotoxicity by this compound. Primary rat hepatocyte cultures incubated for 18 hours with NITC (0-100μM) produced cytotoxicity at concentrations greater than 25 μM (28). The cytotoxicity of these compounds appears to be due entirely to the parent ITC compounds; any cytotoxicity of cysteine or mercapturic acid conjugates likely occurs due to the hydrolysis of the conjugated metabolite back to the parent compound (29).

The cytotoxicity of ITCs may differ depending on the cell line. There are a number of other possible reasons for the differences in IC_{50} values observed in this study in MCF-7/Adr and MCF-12A cells, besides differences in intrinsic sensitivity to the ITCs. The human breast cancer cell line, MCF-7/Adr, has pronounced expression of p-glycoprotein, which may result in lower intracellular concentrations of the ITCs in MCF-7/Adr cells if the ITCs are substrates for P-glycoprotein. Differences in intracellular concentrations in these two cell lines may also occur due to differences in protein binding of the ITC in the cell media. In a study by Xu and Thornalley, it was found that in the absence of fetal bovine serum (FBS), the IC_{50} of PEITC in HL-60 cells was lower compared to the increased IC_{50} values in the presence of 10% and 25% FBS in the media (30). The MCF-7 media used in this investigation contained 10% FBS while the MCF-12A media contained 5% horse serum. If the ITCs are protein bound, lower free concentrations would be present in the medium of MCF-7 cells and therefore available to diffuse across cell membranes in MCF-7 cells. Preliminary studies in our laboratory have indicated extensive protein binding of PEITC in rat serum (Y. Kuo and M.E. Morris, unpublished results). The intracellular accumulation of ITCs has been shown to be highly dependent on intracellular glutathione concentrations; differences in GSH and GST activity in the two cell lines could result in different degrees of accumulation at the same media ITC concentration. Intracellular levels of ITCs will also be dependent on drug metabolism within these cells, which may differ substantially.

The IC_{50} values for the ITCs remained the same in both MCF-7/Adr and MCF-12A cells following 6- and 48-hour exposure times. This contrasted with our findings with DNM and VBL where the IC_{50} values were significantly lower following a 48-hour exposure. Although the reason for this difference is unknown, this may be due to the stability and metabolism of ITCs at

37°C in these cells lines. We have found that the half-life for PEITC in buffer at pH 7.4 at room temperature is about 62 hours. The ITCs are rapidly metabolized in vivo, although clearances and half-lives of unchanged ITCs have not been determined. However, it has been reported that the excretion of ITC conjugates in humans is greatest within the first 2-4 hours after food ingestion and drug conjugates are completely eliminated in urine within 24 hours (21, 31) DNM and VBL have half-lives of about 18.5 and 24.8 hours, respectively, which may be considerably longer than t.hat of the ITCs. (32). If there is rapid metabolism of ITCs in these cell lines, one might expect similar toxicities following short and long exposure times, while the IC50's of DNM and VBL might decrease with longer exposure times (33).

In conclusion, we have shown that the ITCs, BITC, PEITC, and NITC, are able to exert cytotoxic effects in a dose- and time-dependent manner. This cytotoxic effect, which is comparable for BITC and PEITC, with that of DNM, may represent another mechanism important for the chemopreventive effect of the ITCs, or may indicate the potential use of BITC and PEITC as anti-cancer agents. Elucidation of the mechanisms underlying the cancer protective effects of ITCs is of crucial importance, not only for the use of these compounds as chemopreventive agents, but also for the identification or design of more effective chemopreventive agents.

Ackowledgements

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Table 1. IC_{50} of DNM and ITCs in MCF-7/Adr Cells

Compound	Time (hours)	IC_{50} ± SE	N
DNM	2 ***	40.10 ± 1.74	5
	4 ***	34.21 ± 1.32	6
	6 ***	21.87 ± 2.47	6
	48	7.12 ± 0.42	8
VBL	2 *	9.99 ± 0.803	2
	4	5.87 ± 0.469	2
	6	6.99 ± 0.787	2
	48	0.106 ± 0.004	2
BITC	1 ***	14.45 ± 0.28	4
	2 ***	10.37 ± 0.31	4
	3 ***	7.37 ± 0.16	4
	6 ***	5.55 ± 0.19	4
	48	5.95 ± 0.10	2
PEITC	1 ***	20.36 ± 0.78	4
	2 ***	13.98 ± 0.54	4
	3 ***	12.82 ± 0.65	4
	6 ***	7.56 ± 0.31	4
	48	7.32 ± 0.25	4

Table 2. IC_{50} of DNM and ITCs in MCF-12A Cells

Compound	Time (hours)	IC_{50} ± SE	N
DNM	48	N/A	6
BITC	I48	8.07 ± 0.293	4
PEITC	48	7.71 ± 0.074	4
NITC	48	33.63 ± 1.69	4
SULFORAPHANE	48	40.46 ± 1.67	4

Asterisks represents significance from 48 hours -- *$p<0.01$, **$p<0.05$, ***$p<0.001$

Figure Legends

Figure 1. DNM cytotoxicity in MCF-7/Adr cells.

The effect of varying concentrations of DNM on cell growth of MCF-7/Adr cells following exposure times of (■) 2 hours, (▲) 4 hours, (▼) 6 hours and (●) 48 hours. Each data point represents mean±SE from 4 wells in one representative study. The study was repeated five to eight times.

Figure 2. VBL cytotoxicity in MCF-7/Adr cells.

The effect of varying concentrations of VBL on cell growth of MCF-7/Adr cells following exposure times of (■) 2 hours, (▲) 4 hours, (▼) 6 hours and (●) 48 hours. Each data point represents mean±SE from 4 wells in one representative study. The study was repeated twice.

Figure 3. BITC cytotoxicity in MCF-7/Adr cells.

The effect of varying concentrations of BITC on cell growth of MCF-7/Adr cells following exposure times of (▼) 1 hour, (■) 2 hours, (▲) 3 hours, (◆) 6 hours and (●) 48 hours. Each data point represents mean±SE from 4 wells in one representative study. The study was repeated two to four times.

Figure 4. PEITC cytotoxicity in MCF-7/Adr cells.

The effect of varying concentrations of PEITC on cell growth of MCF-7/Adr cells following exposure times of (▼) 1 hour, (■) 2 hours, (▲) 3 hours, (◆) 6 hours and (●) 48 hours. Each data point represents mean±SE from 4 wells in one representative study. The study was repeated four times.

Figure 5. NITC cytotoxicity in MCF-7/Adr cells.

The effect of varying concentrations of NITC on cell growth of MCF-7/Adr cells following exposure times of (▼) 1 hour, (■) 2 hours, (▲) 3 hours, (◆) 6 hours and (●) 48 hours. Each data point represents mean±SE from 4 wells in one representative study. The study was repeated two to four times.

Figure 6. DNM cytotoxicity in MCF-12A.

The effect of varying concentrations of DNM on cell growth of MCF-12A cells following exposure times of (●) 48 hours was measured. Each data point represents mean±SE from 4 wells in one representative study. The study was repeated six times.

Figure 7. BITC cytotoxicity in MCF-12A cells.

The effect of varying concentrations of BITC on cell growth of MCF-12A cells following exposure time of (●) 48 hours. Each data point represents mean±SE from 4 wells in one representative study. The study was repeated four to six times.

Figure 8. PEITC cytotoxicity in MCF-12A cells.

The effect of varying concentrations of PEITC on cell growth of MCF-12A cells following exposure time of (●) 48 hours. Each data point represents mean±SE from 4 wells in one representative study. The study was repeated four to ten times.

Figure 9. NITC cytotoxicity in MCF-12A cells.

The effect of varying concentrations of NITC on cell growth of MCF-12A cells following exposure time of (●) 48 hours. Each data point represents mean±SE from 4 wells in one representative study. The study was repeated four to eight times.

Figure 10. Effect of exposure time to PEITC, BITC, NITC and DNM on IC_{50} values for these compounds in MCF-7/Adr cells.

Figure 1

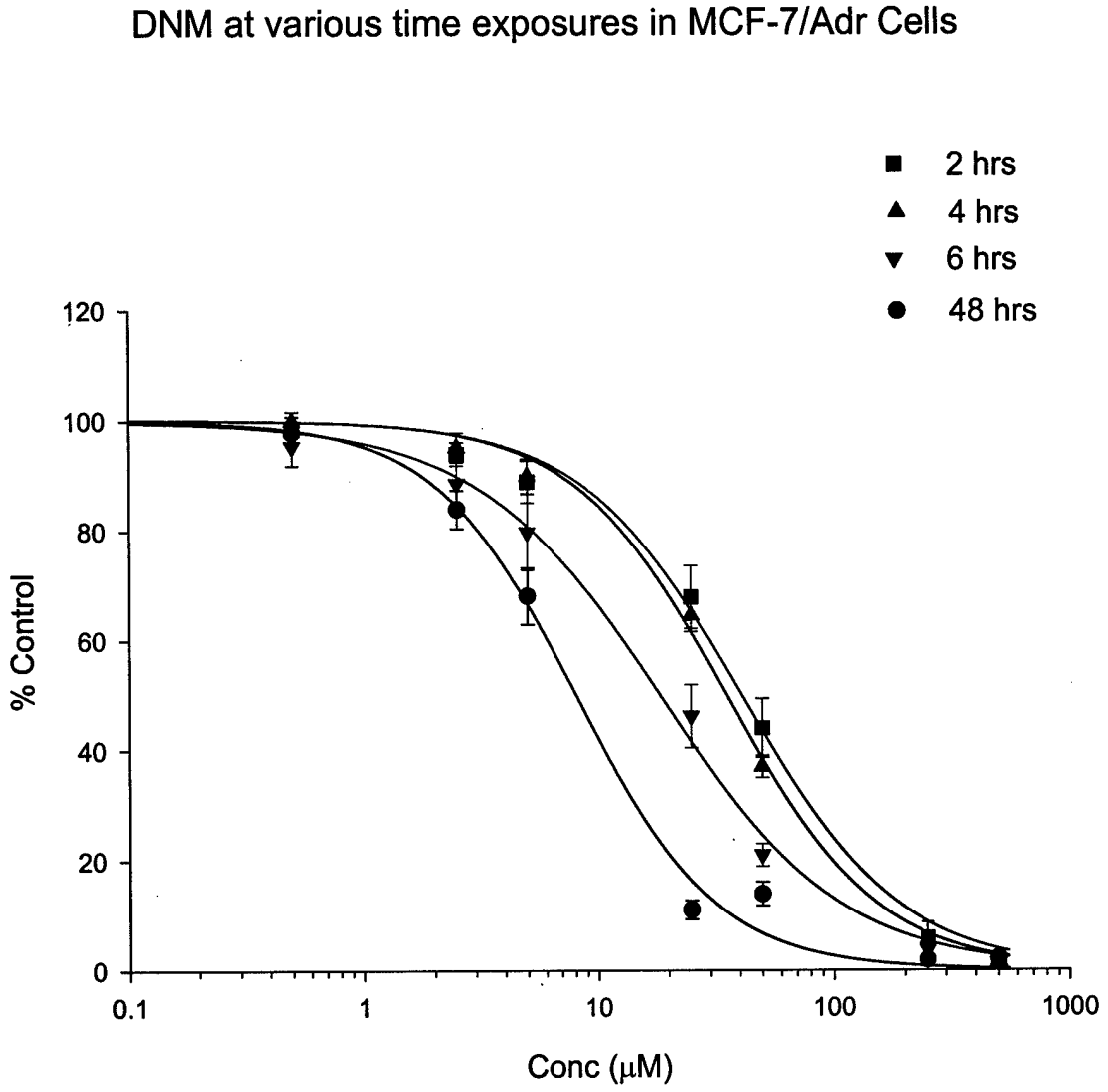

DNM at various time exposures in MCF-7/Adr Cells
2 hrs
4 hrs
6 hrs
48 hrs
% Control
120
100
80
60
40
20
0
0.1
1
10
100
1000
Conc (μM)

Figure 2

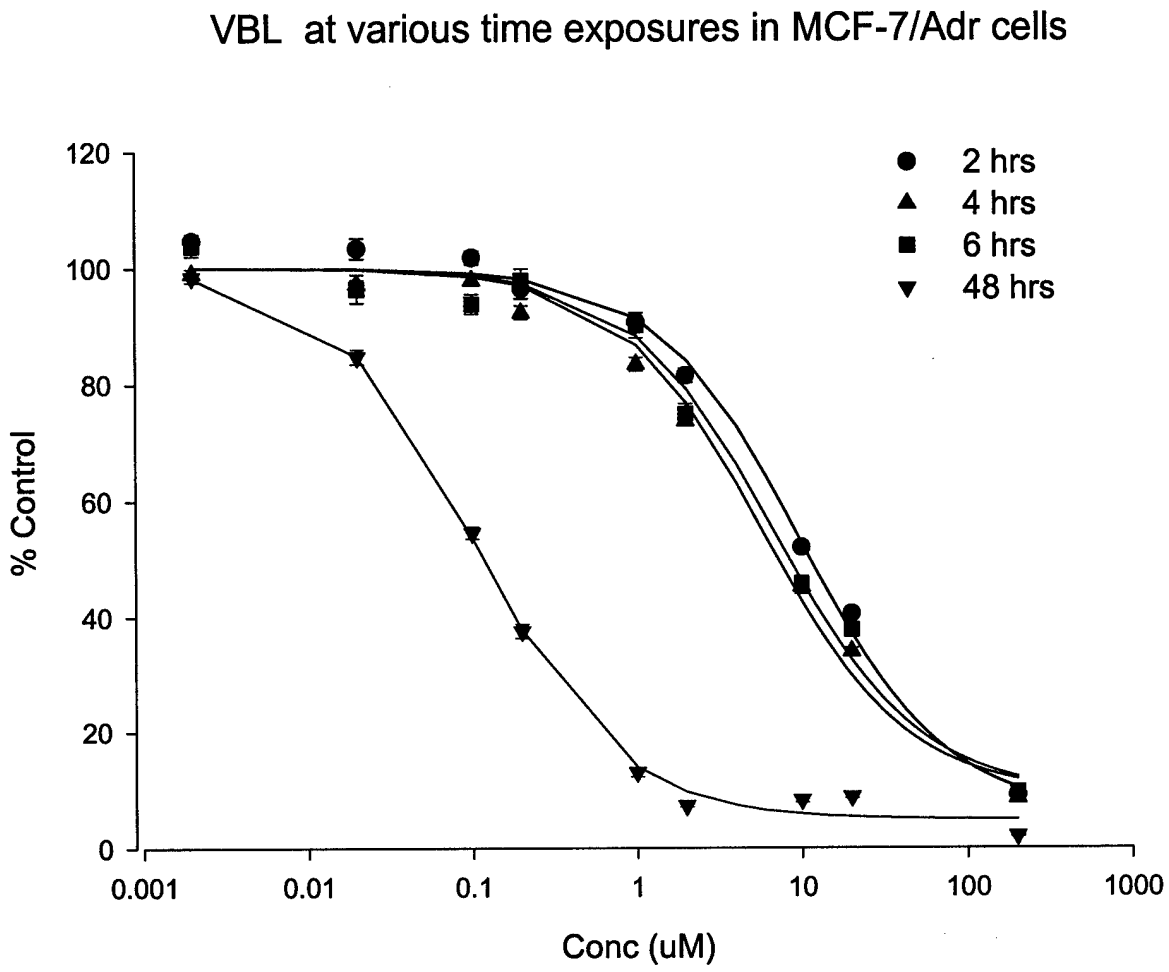

VBL at various time exposures in MCF-7/Adr cells
2 hrs
4 hrs
6 hrs
48 hrs
% Control
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
1000
Conc (uM)

Figure 3

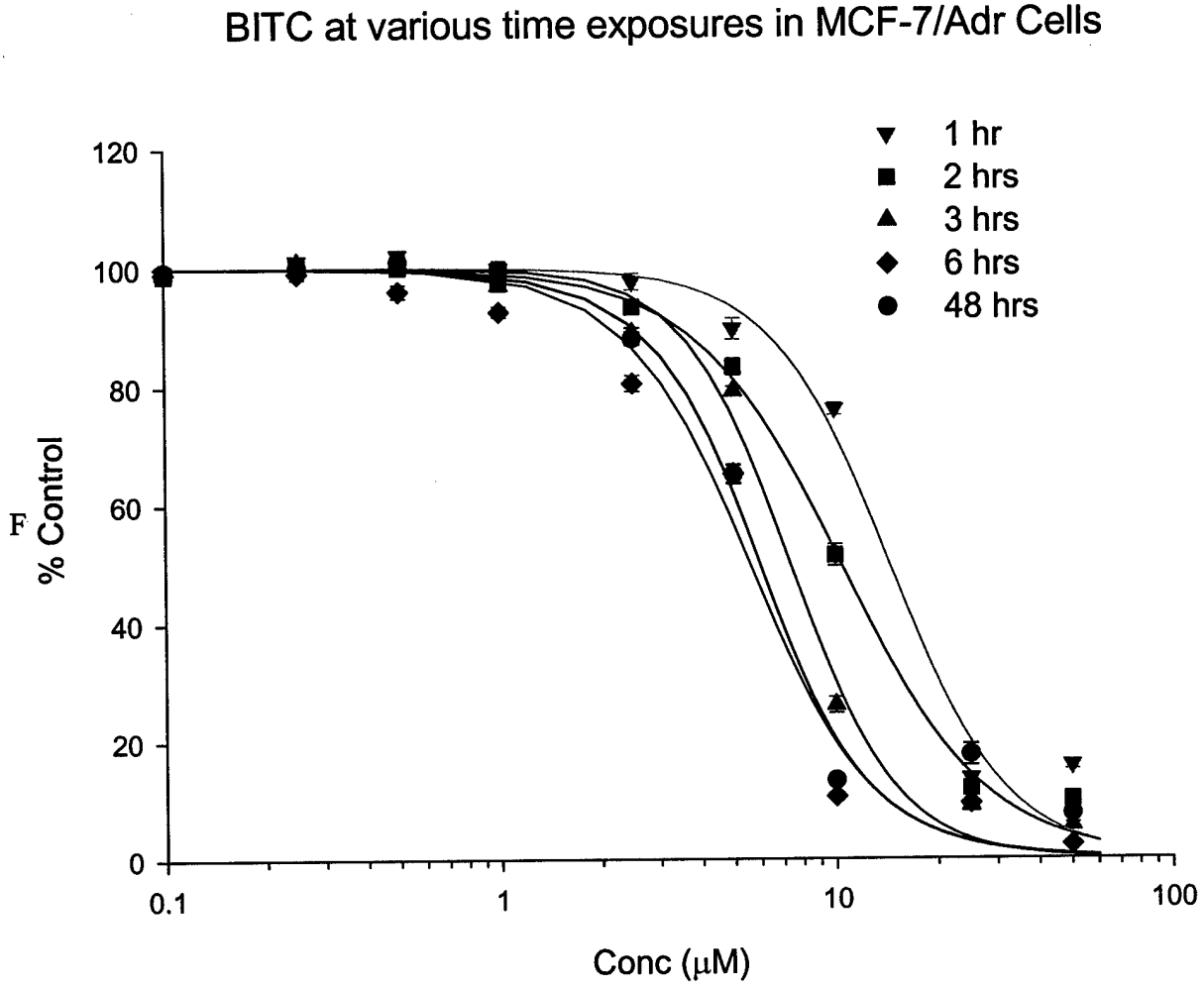

BITC at various time exposures in MCF-7/Adr Cells
1 hr
2 hrs
3 hrs
6 hrs
48 hrs
120
100
80
60
40
20
0
F % Control
0.1
1
10
100
Conc (μM)

Figure 4

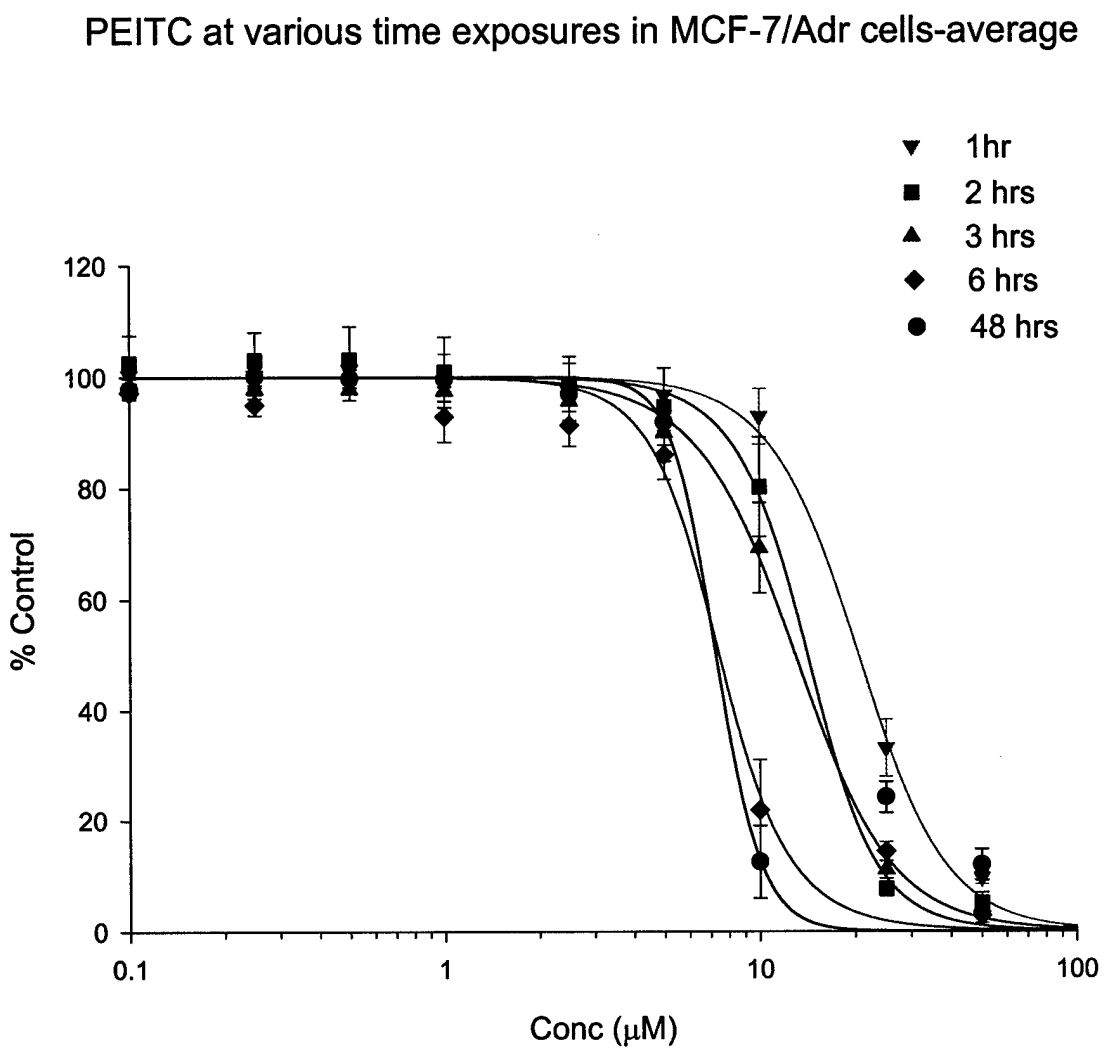

PEITC at various time exposures in MCF-7/Adr cells-average
1hr
2 hrs
3 hrs
6 hrs
48 hrs
120
100
80
60
40
20
0
% Control
0.1
1
10
100
Conc (μM)

Figure 5

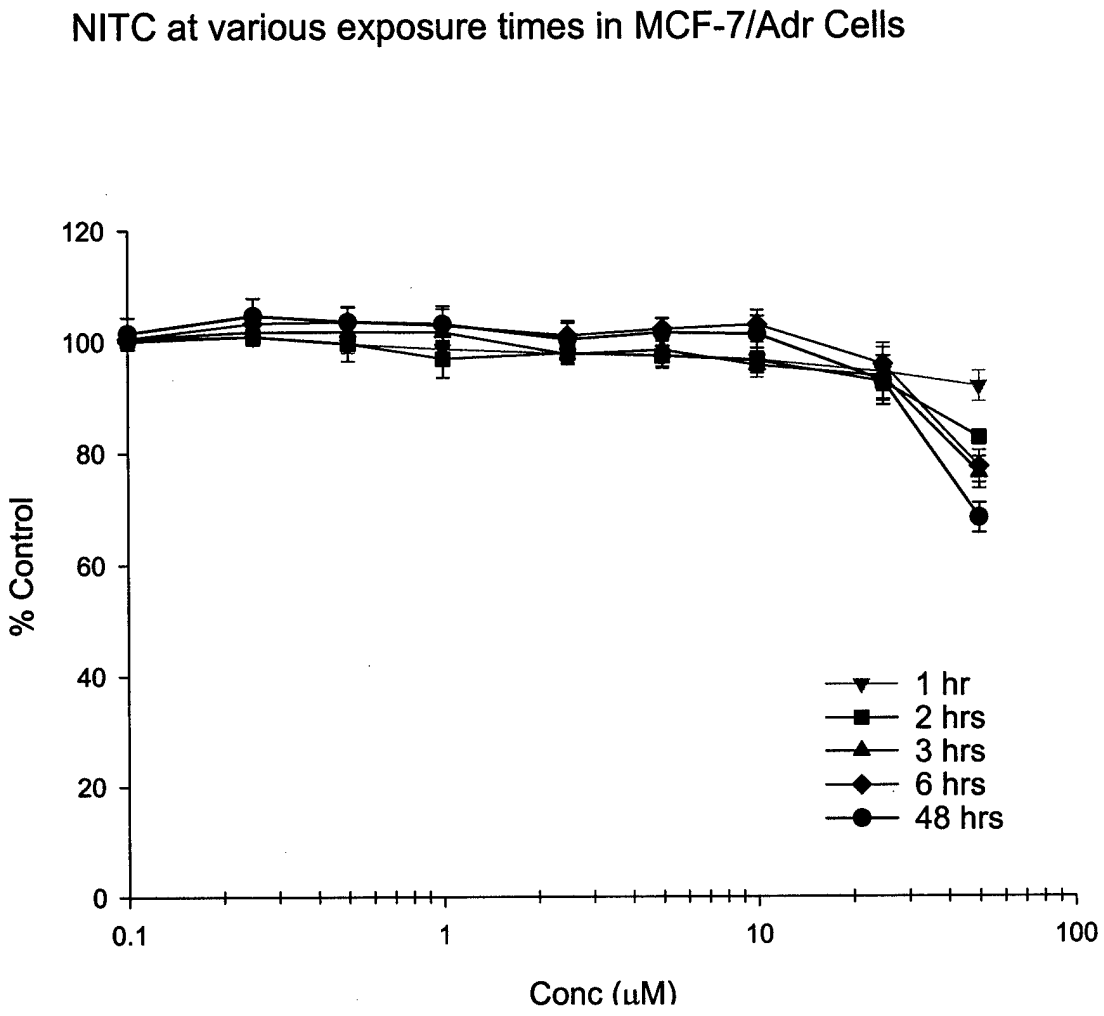

NITC at various exposure times in MCF-7/Adr Cells
120
100
80
60
40
20
0
% Control
0.1
1
10
100
Conc (uM)
1 hr
2 hrs
3 hrs
6 hrs
48 hrs

Figure 6

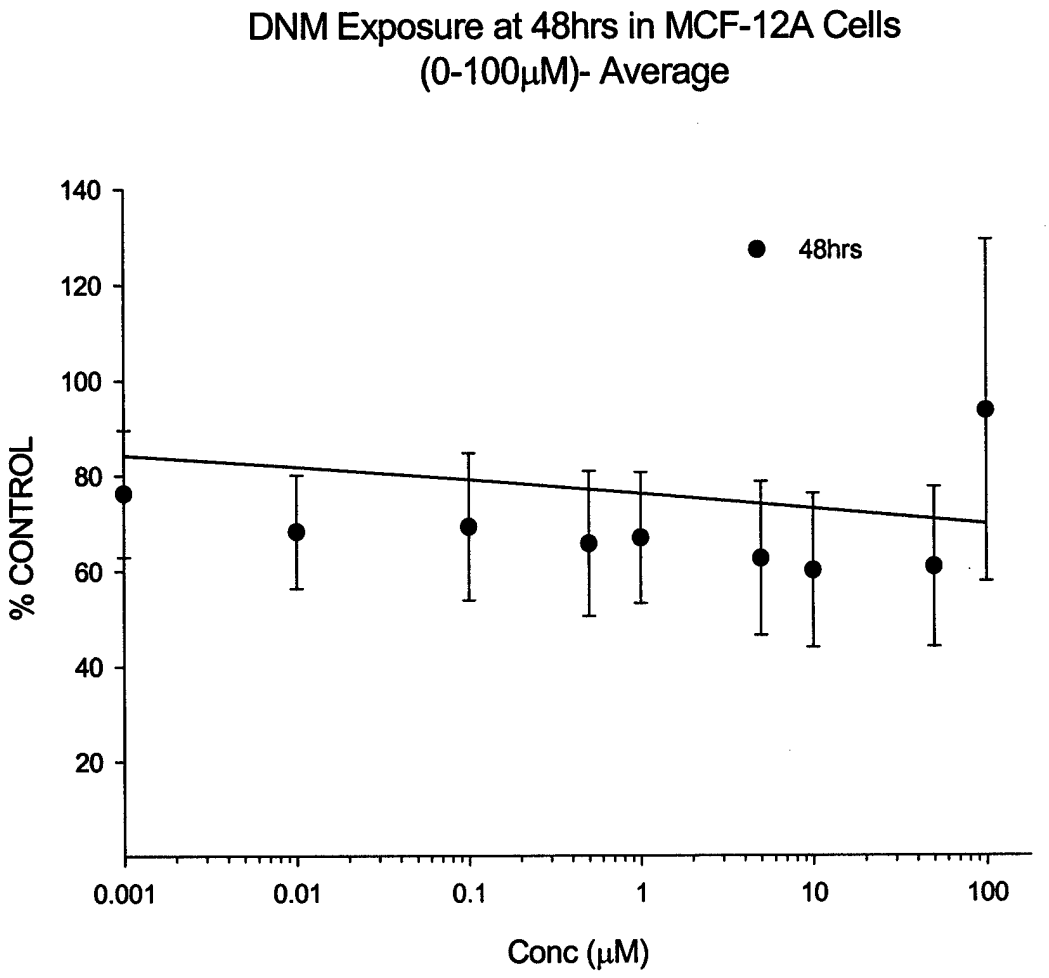

DNM Exposure at 48hrs in MCF-12A Cells
(0-100μM)- Average
48hrs
% CONTROL
140
120
100
80
60
40
20
0.001
0.01
0.1
1
10
100
Conc (μM)

Figure 7

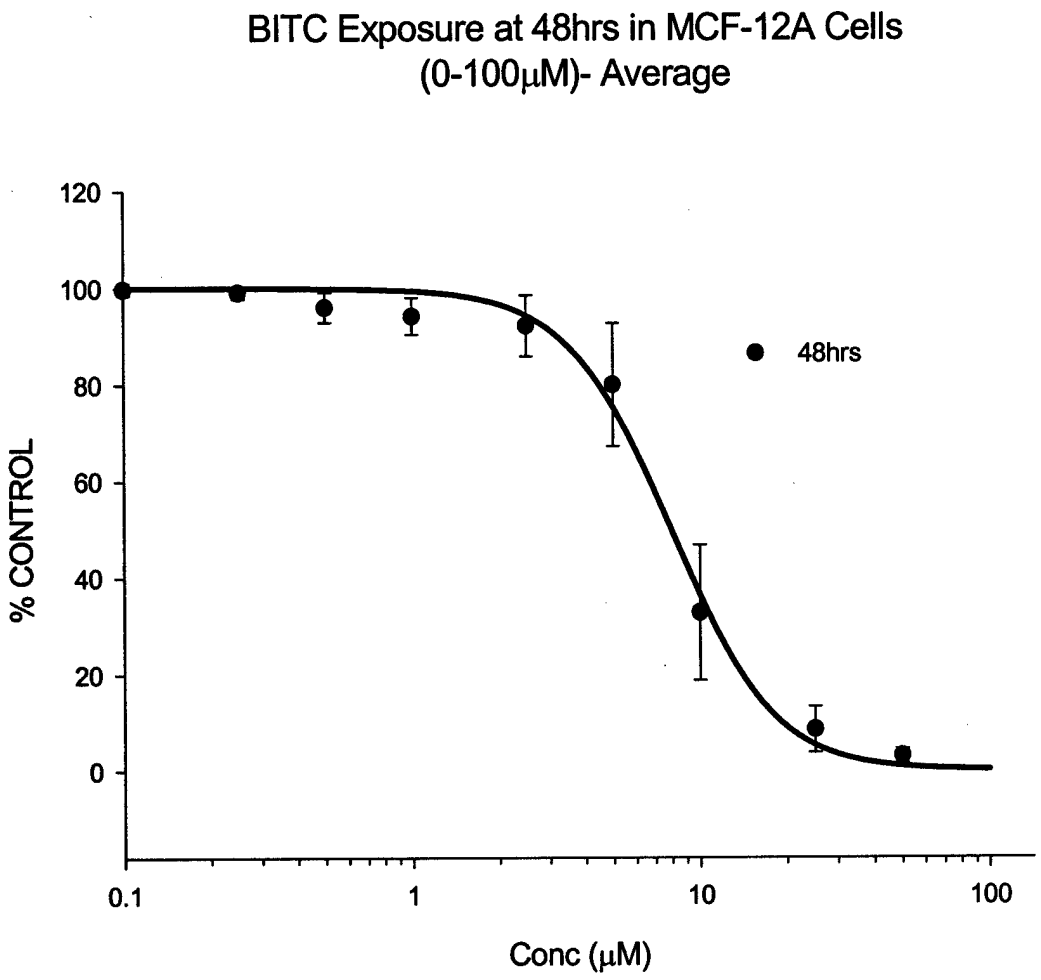

BITC Exposure at 48hrs in MCF-12A Cells
(0-100μM)- Average
120
100
80
60
40
20
0
% CONTROL
48hrs
0.1
1
10
100
Conc (μM)

Figure 8

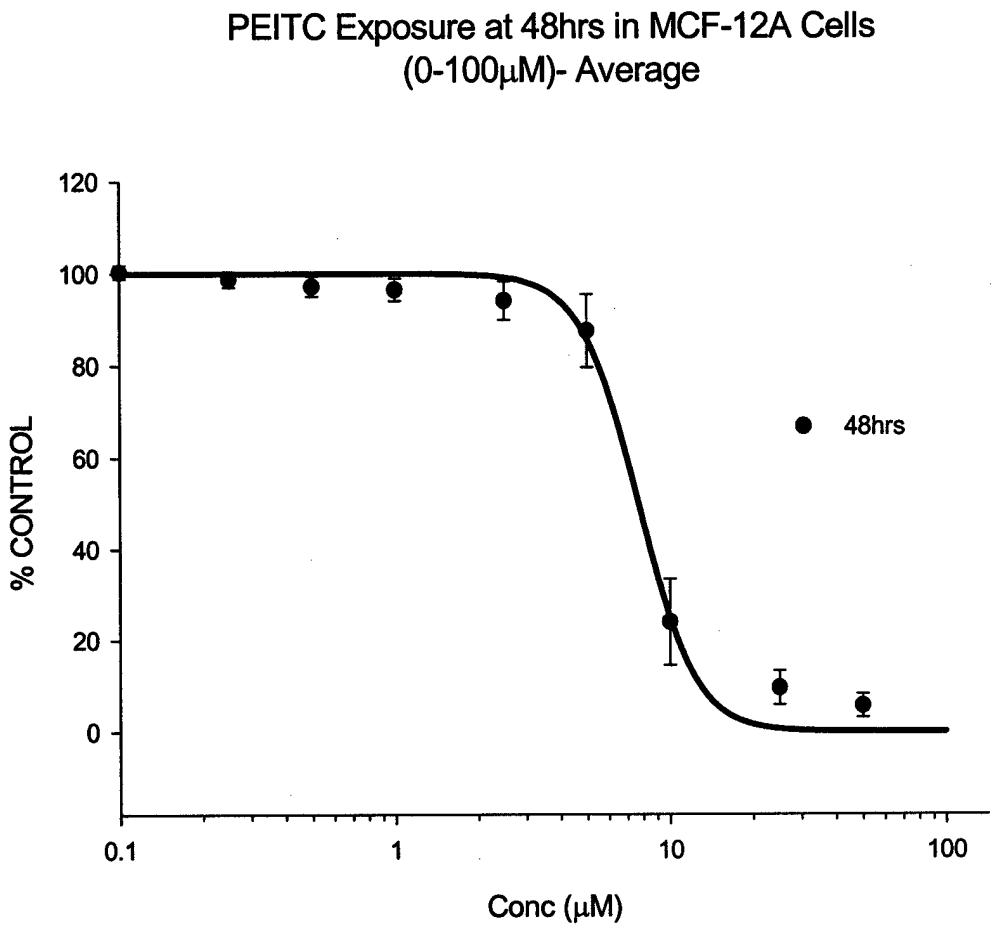

PEITC Exposure at 48hrs in MCF-12A Cells
(0-100μM)- Average
120
100
80
60
40
20
0
% CONTROL
48hrs
0.1
1
10
100
Conc (μM)

Figure 9

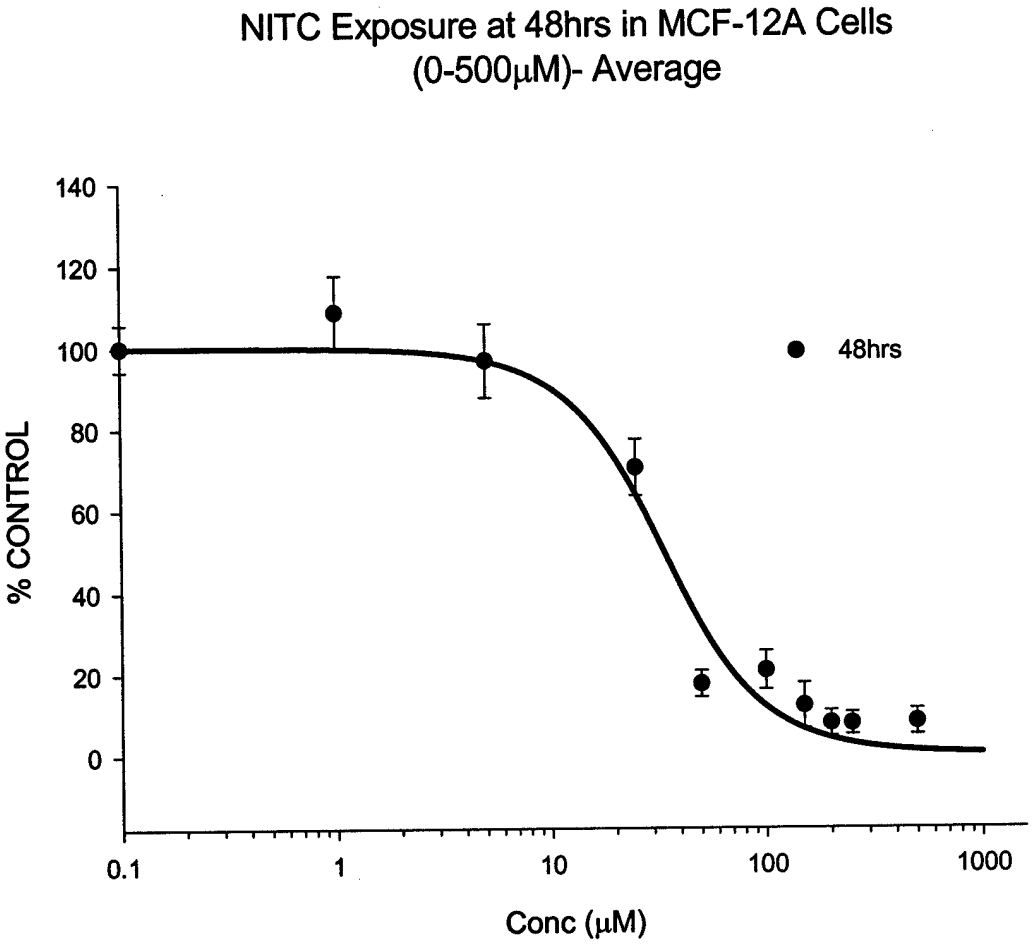
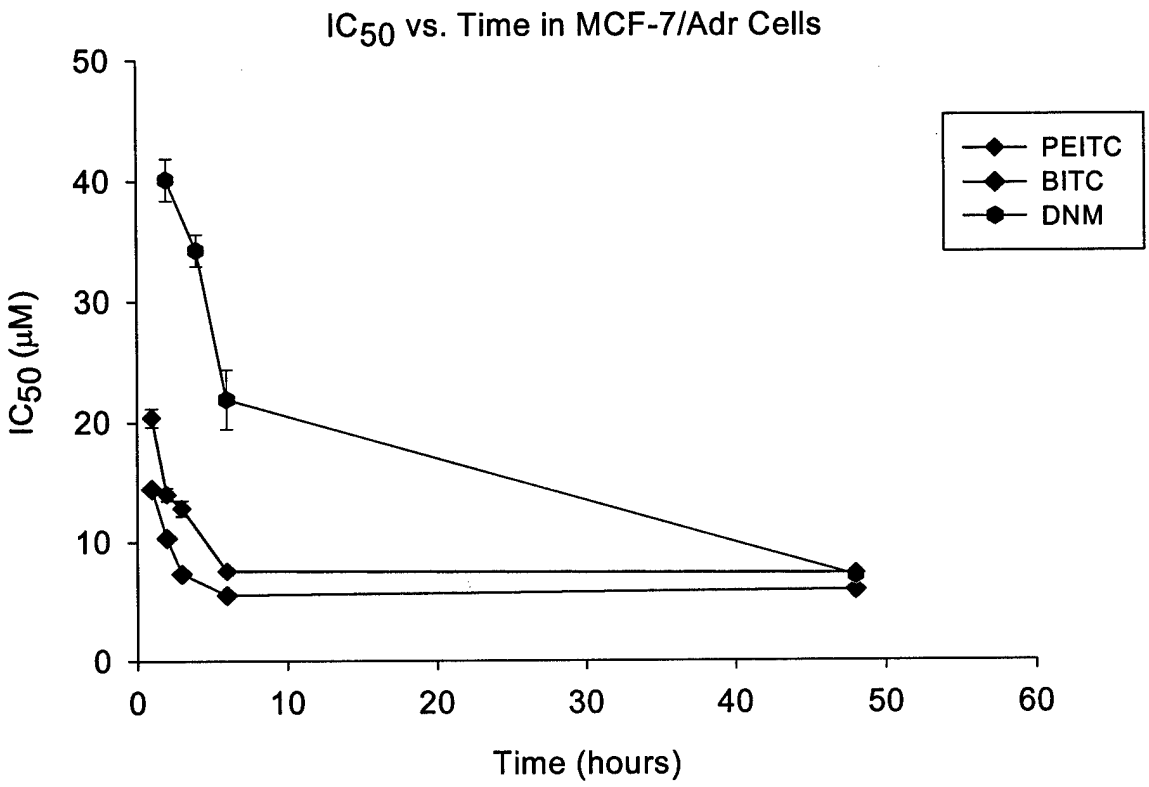

NITC Exposure at 48hrs in MCF-12A Cells
(0-500μM)- Average
140
120
100
80
60
40
20
0
% CONTROL
48hrs
0.1
1
10
100
1000
Conc (μM)

Figure 10


IC50 vs. Time in MCF-7/Adr Cells
IC50 (μM)
Time (hours)
PEITC
BITC
DNM
0
10
20
30
40
50
60

Protocol for preparation of [^{14}C]Phenethyl-isothiocyanate

[^{14}C]Phenethyl-isothiocyanate in my study will be prepared by a three-step synthesis. The preparation is performed in the hood in Hochstetter 539. The starting material is [^{14}C] KCN (25mCi), and the two intermediates are [^{14}C] benzylcyanate and [^{14}C] phenethylamine. The final product is [^{14}C]phenethyl-isothiocyanate.

1. The synthesis of [^{14}C] benzylcyanate:

25mCi [^{14}C] KCN (0.5 mmol) and 2.0 mmol KCN is dissolved in 0.2 ml distilled water in a 25 ml round-bottom reaction flask. Benzylchoride (2.4mmol) in 2.5 ml DMSO is added to the KCN solution with stirring. The mixture is heated on a water bath at 70-80 °C for 2 hours. After cooling to room temperature, 5ml distilled water is added, and a pasteur pipette is used to transfer the mixture from the reaction vial to a 15ml screw-cap test tube. The mixture is extracted twice using 3ml aliquots of dichloromethane. The extracted organic phases are combined and put into a 15ml screw-cap test tube. Note, there are no centrifugation steps in this protocol. After rinsing with distilled water 3ml, the extracted organic phase is dried over anhydrous sodium sulfate or magnesium sulfate for 2 hr. and filtered through cotton (the filter is made of a pasteur pipette with cotton at the tip). When filtrating, the test tube will be placed in a beaker covered with filter paper to secure the tube and to catch any possible leakage). The solution is then put into a dry test tube and dichloromethane is removed by dry N_2. An oil-like liquid, which has been identified as [^{14}C]-benzylcyanate, is obtained. The yield of this step is about 80-90%, based on our cold synthesis. The aqueous waste generated in this step is about 6-8 ml and will contain the 40% radioactivity (~5 mCi) lost in this step of the synthesis. The dry waste generated in this step should contain only small amounts of radioactivity.

2. The synthesis of [^{14}C] Phenethyl amine:

[^{14}C] Benzylcyanate (~1.8mmol) is dissolved in 0.5 ml dichloromethane and transferred to a two-neck flask. Then evacuate the flask and refill with dry nitrogen. Use a syringe to transfer 4 ml borane in THF (1M) to the reaction flask and add it dropwisely while stirring. The reaction mixture is refluxed for 0.5 hr (Heated by a water bath). After cooled to room temperature, 0.2 ml HCl (5N) is added dropwisely into the mixture with stirring. Then the mixture is heated for 0.5 hr at 50-60 °C (water bath covered with aluminum foil to avoid evaporation). Use 1N sodium hydroxide to adjust the pH value to 11-12. (The pH paper used will be discard as radioactive waste). Extract the mixture with ethyl ether 2ml for three times. The ether phase is combined and dried over sodium sulfate or magnesium sulfate for 2 hr. The solution is filtered (as described above). The ether solvent is removed by a stream of dry nitrogen. An oily liquid is obtained, which is identified as [^{14}C] phenethyl amine. The yield of this step is 62% or greater. The aqueous waste generated in this step is about 6-8 ml and contain about 5 mCi radioactivity. The dry waste generated in this step should contain only small amounts of radioactivity.

The synthesis of [^{14}C] phenethyl-isothiocyanate:

Dissolve the [^{14}C] phenethyl amine (~0.5mmol) in 1ml dichloromethane and transfer to a round bottom reaction vial using a pasteur pipette and rinse the tube with 0.5 ml dichloromethane. 1ml of NaOH (1N) is added with stirring at 0 °C (ice bath). 400µl thiophosgene solution (1.0 M in dichloromethane) is added dropwisely. The mixture is

stirred at 0°C for 15 min.in a ice bath (same water bath as above, but ice is added). The reaction is stopped and the two phases separated. The aqueous phase is extracted with 2.5 ml dichloromethane twice. The organic phase is combined together and dried over sodium sulfate or magnesium sulfate for 2 hr. After filtration (as described above) and removal of solvent by dry nitrogen, ~0.3 mmol crude product is obtained. The yield will be 60% or greater. The aqueous waste generated in this step is about 6-8 ml and will contain the rest of 40% radioactivity (~3-5mCi). The dry waste generated this step should contain little radioactivity.

Further purification is done by a silica column using hexane 10 ml. 1 ml-fractions will be collected and fractions 2-4 combined. Evaporate the hexane by dry nitrogen and an oily liquid will be obtained. NMR will be used to identify the product and determine the purity.

Full Paper for *J Chromatogr B: Biomed Appl*

Determination of α-naphthylisothiocyanate (1-NITC) and metabolites α-naphthylamine (1-NA) and α-naphthylisocyanate (1-NIC) in rat plasma and urine by high-performance liquid chromatography

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Abstract

A rapid and sensitive high-performance liquid chromatographic (HPLC) assay for determination 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 chromatographic analysis was carried out using reversed-phase isocratic elution with a Partisphere C-18 5 μm column, a mobile phase acetonitrile/water (ACN:H_2O 70:30, v/v), and detection by ultraviolet (UV) absorption at 305 nm. 1-NITC and 1-NA were found in rat plasma and urine samples, respectively, with retention time 5.9 and 2.2 min by elution of. 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. The standard curve was linear over a concentration range of 10-5000 ng/ml for 1-NITC in plasma and 100-50000 ng/ml for 1-NA in urine ($r > 0.999$). At low (10 ng/ml), medium (500 ng/ml), and high (5000 ng/ml) concentrations of quality control samples (QCs), the within-day and between-day accuracy were 95-106% and 97-103% for 1-NITC in plasma, respectively. The values for within- and between-day precision were 97-100% and 93-97%, respectively. For 1-NA in urine, the within- and between-day accuracy and precision values were 96-106% and 97-99%, respectively. ACN extraction of plasma and urine samples resulted in recoveries of 1-NITC between 93 and 97%, and of 1-NA between 95 and 110%. The stabilities of 1-NITC, 1-NA, and 1-NIC for time course were studied in rat plasma, urine, ACN extracts of plasma and urine, ACN, and universal buffer (citrate-phosphate-borate-HCl, pH 2-12) at room temperature (RT), 4, -20, and -80°C over 96 h. 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 at RT, 1-NITC degraded rapidly at pH values ranging from 2 to 12. 1-NA was stable in all tested matrix at all temperatures. 1-NIC was unstable in plasma, urine, and ACN extracts of 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.

The validated HPLC assay was used in a pharmacokinetic study of 1-NITC following intravenous (i.v.) bolus administration of 25 mg/kg dose to a female Spraque-Dawley rat. 1-NITC and 1-NA were detected and quantified in rat plasma and urine. Based on noncompartmental analysis, values for 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.

Keywords: α-naphthylisothiocyanate (1-NITC); α-naphthylamine (1-NA); α-naphthylisocyanate (1-NIC); HPLC; metabolite; pharmacokinetics

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 [1-2]). α-naphthylisothiocyanate (1-NITC) (Fig. 1) was reported as a carcinogenesis inhibitor in rats as early as 1960's [3-8]. Other research has focused on the hepatotoxicity of 1-NITC [9-11]; high doses of 1-NITC produces hepatic lesion resembling those occurring in biliary cirrhosis in humans. This has led to the use of 1-NITC as a model chemical to study human cirrhosis and the mechanism involved in drug-induced cholestasis.

Recently, we have found that 1-NITC increases the sensitivity of multidrug resistant breast cancer MCF-7/Adr cells, a cell line with P-glycoprotein (P-gp) overexpression, to the anticancer drugs daunomycin (DNM) and vinblastine (VBL). Additionally, 1-NITC did not show cytotoxicity against several human breast cancer or normal mammary cell lines *in vitro* even at the highest test concentration of 100 μM (Tseng and Morris, unpublished data). These findings indicated that 1-NITC could be potentially used to overcome P-gp-mediated multi-drug resistance (MDR) to anticancer drugs.

In the past decades, 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].

To our knowledge, the pharmacokinetics of 1-NITC and its metabolites in rat plasma and urine has never been studied before. Moreover, no systematic validation studies for 1-NITC and its metabolites

have been reported by HPLC assay in rats. Therefore, in this paper, we report the development and validation of a rapid and sensitive HPLC assay able to detect parent drug 1-NITC and its metabolites in rat plasma and urine for future pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

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

2.2. Preparation of rat plasma and urine samples for calibration standards and 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.

The working 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 NE work solution (0.5 mg/ml), 5 μl appropriate 1-NITC working solution, and added 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, 10000, 20000, and 50000 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 50000 ng/ml for 1-NA), respectively, were prepared by the same procedures as standards above.

2.3. HPLC instrumentation and conditions

The Waters HPLC system (Milford, MA) consisted of a 1525 binary pump, a 717plus autosampler (a 250-μl injector and a 200-μl loop) configured with a heater/cooler, a Model 5HC column oven, and a 2487 UV detector. The column and autosampler temperatures were kept at room temperature (21 ± 1°C) and 4°C, respectively. The reversed-phased chromatography was performed with a stain-steel less

Whatmann (Clifton, NJ) Partisphere C-18 5 μm column 125 × 4.6 mm i.d. protected by a guard column, 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 single wavelength 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 linear range of calibration curve. LLQ was defined as the concentration at the lowest QC samples producing an assayed concentration within 10% of the theoretical value (i.e. accuracy between 90 and 110%) and yielding precision 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. The assay was judged linear if the correlation coefficient r was more than 0.999.

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 nominal 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 extraction recovery of 1-NITC and 1-NA was established at QCs by comparing peak area ratios (1-NITC/NE and 1-NA/NE) of samples prepared in blank plasma and urine samples with those for non-processed samples prepared in ACN. The mean recoveries at low, medium, and high concentrations were determined by within- and between-day analysis.

2.5. Stability

The stability of 1-NITC, 1-NA, and 1-NIC was studied in different matrices of rat plasma, urine, ACN extracts of plasma and urine, ACN, and universal buffer (citrate-phosphate-borate-HCl, pH 2-12) at four designated temperature over 96 h. 1-NITC, 1-NA, or 1-NIC (200 ng/ml as final concentration), along with internal standard NE (10 μg/ml), was spiked in plasma and ACN, respectively, for stability in plasma, ACN extracts of plasma, and ACN samples at room temperature (RT), 4, -20, and -80°C (with data points at 0, 1, 3, 6, 7, 9, 18, 24, 42, 48, 64, 72, and 96 h for 1-NITC in plasma samples; at 0, 4, 8, 24, 48, 72, and 96 h for 1-NITC in ACN extracts of plasma and ACN samples; at 0, 9, 18, 24, 48, 72, and 96 h for 1-NA in plasma samples and ACN extracts of plasma; at 0, 4, 8, 24, 48, 72, and 96 h for 1-NA in ACN samples; at 0, 4, 8, 24, 48, 72, and 96 h for 1-NIC in ACN samples). The stability of 1-NITC, 1-NA, and 1-NIC in urine and ACN extracts of urine were tested at similar time intervals (with data points at 0, 1, 3, 6, 7, 9, 18, 24, 42, 48, 64, 72, and 96 h for 1-NITC in urine samples; at 0, 4, 8, 24, 48, 72, and 96 h for 1-NITC in ACN extracts of urine; at 0, 9, 18, 24, 48, 72, and 96 h for 1-NA in urine samples; at 0, 4, 8, 24, 48, 72, and 96 h for 1-NA in ACN extracts of urine samples), except final concentration of tested compounds and internal standard (i.e. 10 μg/ml for 1-NITC, 1-NA, and 1-NIC; 50 μg/ml for NE). The stability of1-NITC, 1-NA, and 1-NIC in universal buffer was carried out in a broad pH range from 2 to 12 at RT (with data points at 0, 2, 4, 8, 16, 24, 30, 36, 48, 60, 72, 84, and

96 h) with same concentration as in plasma. The compound is considered stable if the variation of quantitation is less than 10% (i.e. 90-110% of 0-h data).

2.6. 1-NITC pharmacokinetics in rat

The jugular vein cannula was inserted into a female Sprague-Dawley (Harlan, Indianapolis, IN) rat (200-225 g) following an i.m. injection of ketamine 90 mg/kg and xylazine 10 mg/kg (Henry Schein, Melville, NY). 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), 10% cremophor EL (Sigma), and 80% sterile saline (Braun Medical, Irvine, CA) solution was administered as an intravenous (i.v.) bolus through the cannula.

The blood samples (250 μl each) were collected at 5, 10, 20, 30 min, 1, 2, 4, 6, 9, 12, and 24 h following administration, and placed in heparinized 0.6-ml microcentrifuge tubes. The plasma were immediately separated from blood samples via centrifugation at 1,000 *g* for 10 min at 4°C and stored at -80°C to prevent potential degradation of compounds. Fifty microliter (50 μl) of each plasma sample was spiked with 5 μl NE (0.5 mg/ml), and followed same procedures as calibration standards and QCs of 1-NITC. The non-compartment fitting was obtained by WinNonLin version 2.1 (Pharsight,Cary, NC).

The urine samples were accumulated at 2, 4, 6, 9, 12, 24, and 25 h time points, and the volume was measured. After spiked with 0.1% sodium azide (Fisher), the urine samples were centrifuged at 1,000 *g* for 10 min at 4°C and stored at -80°C to prevent potential degradation of compounds. Fifty microliter (50 μl) of each urine sample was spiked with 5 μl NE (2.0 mg/ml), and followed same procedures as calibration standards and QCs of 1-NA.

3. Results

Specificity and selectivity

Figs. 2 and 3 display typical chromatographs resulting from HPLC analysis of the extracts of rat plasma and urine. Blank rat plasma and urine do not demonstrate any interference peaks (Figs. 2a and 3a). The spiked mixture of three interested compounds (200 ng/ml each) and internal standard in ACN solution are well separated each other with retention time (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 disappearance of 1-NIC due to possible rapid degradation in plasma and urine samples (Figs. 2d and 3c). Also, 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, 2c, 2d, 3a, 3b, and 3c). In biological samples for pharmacokinetic studies, 1-NITC and 1-NA were the only compounds that could be detected in plasma (Fig. 2e) and urine (Fig. 3d), respectively.

Lower limit of quantitation (LLQ)

The LLQ of 1-NITC, 1-NA, and 1-NIC were tested 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.

Linearity

The linear regression correlation coefficients r was more than 0.999 in every single run (data not shown). The linearity for 1-NITC and 1-NA was over a 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 could be linear 30-5000 ng/ml and 100-50000 ng/ml, respectively. The data not shown here are available upon request.

Accuracy, precision and recovery

As shown in Table 2, at low (10 ng/ml), medium (500 ng/ml), and high concentration (5000 ng/ml) levels, the within- and between-day accuracy were 95-106% and 97-103% for 1-NITC, respectively. The within- and between-day precision were 97-100% and 93-97%, respectively. Moreover, the protein precipitation with ACN for plasma samples resulted in their recoveries between 93 and 97% for both within- and between-day analysis.

As shown in Table 3, at low (100 ng/ml), medium (5000 ng/ml), and high concentration (50000 ng/ml) levels of 1-NA, the within- and between-day accuracy are 96-106%, precision 97-99%, and recovery 95-110%.

Stability

1-NITC was stable at -20°C and -80°C in plasma, urine, ACN extracts of plasma and urine (Figs. 4a-4d), and at all tested temperature in ACN over 96 h (Fig. 4e). However, 1-NITC was degraded at RT and 4°C in plasma, urine, and ACN extracts of plasma and urine (Figs. 4a-4d). The faster degradation at RT than at 4°C indicated a temperature-dependent pattern in each matrix (Figs. 4a-4d). Moreover, the degradation of 1-NITC in plasma (Fig. 4a) and urine (Fig. 4c) are much steeper than the

corresponding ACN extracts of plasma (Fig. 4b) and urine (Fig. 4d) at same temperature (RT and 4°C). The degradation of 1-NITC in ACN extracts of urine (Fig. 4d) was much slower than ACN extracts of plasma (Fig. 4b). When in universal buffer at RT, 1-NITC was degraded with very similar patterns from pH 2 to pH 10 over 96 h (Fig. 4f), except in pH 11 with different pattern (Fig. 4f) and in pH 12 with instant degradation (data not shown). The degradation product of 1-NITC in universal buffer was confirmed to be 1-NA (data not shown). But the degradation product of 1-NITC in plasma, urine, and ACN extracts of plasma and urine has not been identified.

As shown in Figs. 4g-4l, 1-NA was stable in all matrix at RT, 4, -20, and -80°C with quantitation variation less than 10% during individual tested period. In comparison, 1-NIC was rapidly degraded in plasma (Fig. 2d), urine (Fig. 3c), ACN extracts of plasma and urine (data not shown). In universal buffer, 1-NIC was rapidly degraded to form 1-NA (data not shown). However, 1-NIC was stable in ACN over 96 h (Fig. 4m).

Application of assay in rat pharmacokinetic studies

The described analytical method was applied to preliminary pharmacokinetic studies of 1-NITC in rat. The parent drug 1-NITC and metabolite 1-NA were the only compounds that could be detected in plasma and urine samples, respectively, after a dose of 25 mg/kg i.v. bolus administration (Figs. 2e and 3d). The concentration of 1-NITC in plasma over 24 h and 1-NA in urine over 25 h are determined in Tables 4 and 5 by calculation based on calibration curves. The data of Table 4 is plotted in Fig. 5.

4. Discussion

Compared with the published HPLC assay for 1-NITC using an external standard method [17-22], we chose to use an internal standard method to allow simultaneous qualification and quantitation of parent

drug and metabolites, with the advantage of avoiding changes in concentration due to instrumental variation for within- and between-days. Based on the features of the chemical structures (Fig. 1, naphthylene (NE) was selected as an ideal internal standard candidate. Additionly we found that other chemically unrelated compounds, such as acetophenone (AP) and propiophenone (PP), could also be used as the internal standard in this assay (data not shown).

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 least interference by endogenous compounds present in plasma and urine at this wavelength. Under the current HPLC conditions, the LLQ is highly sensitive with 0.5 ng (10 ng/ml) and 1.5 ng (30 ng/ml) for 1-NITC in plasma and urine, and 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 involved the use of a protein precipitation step with ACN (four-fold volume of plasma) followed by centrifugation at 10,000 *g* for 10 min at 4°C. Using protein precipitation was more convenient and time-saving than liquid-liquid extraction (LLE) and solid phase extraction (SPE), and resulted in the least amount of interference with endogenous compounds (Figs. 2a, 2c-2e), while retaining high extraction efficiency (Table 2). Other organic solvents, such as MeOH and acetone, were also investigated in our preliminary studies but produced endogenous interferences and/or variability of recovery.

Since the direct injection of urine supernatant resulted in tailing peaks of 1-NITC, 1-NA, and NE (data not shown), an extraction step for urine samples using ACN, MeOH, acetone, or acetyl acetate (EtOAc) was investigated. The extracts were either injected directly or reconstituted by ACN or

mobile phase. Extraction with ACN followed by centrifugation at 10,000 *g* for 10 min at 4°C resulted in the best accuracy, precision, and recovery (Table 3)(Figs. 3b-3d).

Compared to the amine group in 1-NA, the isothiocyanate group (N=C=S) in 1-NITC and isocyanate group (N=C=O) in 1-NIC are highly active to hydrolysis. Therefore, the stabilities of 1-NITC, 1-NA, and 1-NIC were systematically investigated with regards to matrix and temperature effects during a specific time course. 1-NA was stable in all tested matrix at all tested temperatures (Figs. 4g-4l). However, the stabilities of 1-NITC and 1-NIC varied under different experimental conditions. The stability of 1-NITC was temperature-dependent in plasma, urine, ACN extracts of plasma and urine, i.e. stable at -20 and -80°C but degraded at RT and 4°C (Fig. 4a-4d). Based on this critical information, the plasma and urine samples for pharmacokinetic studies were centrifuged at 4°C and stored at -80°C immediately. The standards of 1-NITC in plasma and urine for calibration curve and QCs were prepared individually on ice and tested immediately at 4°C in autosampler, and the validation and quantitation data (Tables 1, 2, and 4) were reliable since the degradation of 1-NITC was less than 5% within 1 h for plasma samples (Fig. 4a) and within 4 h for ACN extracts of plasma (Fig. 4b) at 4°C.

Moreover, our experiment showed that 1-NITC and 1-NA were stable in plasma and urine at -80°C for more than two months (data not shown). However, in order to prevent potential degradation, samples for pharmacokinetic studies should be analyzed shortly after sampling (e.g. within two weeks). On the other hand, the temperature-independent stability of 1-NITC in ACN indicated that ACN is an ideal extraction solvent for 1-NITC (Fig. 4e). In addition, the pH-independent degradation of 1-NITC in universal buffer further confirmed its high liability to hydrolysis (Fig. 4f). 1-NITC degradation at pH values of 2 to 10 was very similar to that of 1-NITC degradation in plasma and urine at RT (Fig. 4a and 4c).

The isocyanate group (N=C=O) was more reactive than the isothiocyanate group (N=C=S) based on our study results. 1-NIC was instantly degraded in aqueous matrix, i.e. plasma, urine, ACN extracts of plasma and urine, and universal buffer. However, the degradation compound of 1-NIC in plasma, urine, and ACN extracts of plasma and urine, was not identified. However, its degradation compound in universal buffer was confirmed as 1-NA. In addition, the information on 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) at least within 4 min, but probably shorter than 15 min (10 min for sample preparation and 5 min for mobile phase elution) (Fig. 2d). The phenomena was probably responsible for the reason that 1-NIC was not detected in all plasma and urine samples because of the time for its presence *in vivo* and following sample preparation much longer than 15 min.

Using this HPLC assay, 1-NITC and 1-NA were quantified in rat plasma and urine, respectively (Table 4 and 5). Analysis of plasma samples allowed the determination of pharmacokinetic parameters for 1-NITC (clearance of 2.07 l/kg/h, volume of distribution of 14.3 l/kg, and half life of 4.76 h). 1-NITC was not detected in urine samples. The metabolite 1-NA was present in urine samples but the total recovery was low (0.4% of the injected dose of -NITC) (Table 5) in urine indicated that 1-NITC may undergo elimination by other mechanism such as biliary excretion, and the 1-NA may be further metabolized and/or eliminated by other mechanism than renal excretion.

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. The assay provides high sensitivity, with LLQ of 10, 30, and 10 ng/ml for 1-NITC in plasma, urine, and ACN; 30, 100, 30

ng/ml for 1-NA in plasma, urine, and ACN; 30 ng/ml for 1-NIC in ACN. The linear range of calibration curve for 1-NITC was evaluated over a concentration range from 10 to 5000 ng/ml in plasma and 30 to 5000 ng/ml in urine, with linear regression correlation coefficients of greater than 0.999. Similarly, the calibration curves were linear over a range from 30 to 5000 ng/ml in plasma and 100 to 50000 ng/ml in urine for 1-NA. The method was proved precise and accurate, with the within- and between-day precision and accuracy within the acceptable range of 90-110% for QCs at low, medium and high concentration levels. The extraction of 1-NITC from plasma and 1-NA from urine with ACN was efficient due to the absolute recovery 93-97% and 95-110%, respectively. The stability studies showed that 1-NITC was stable at all tested temperature 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 was degraded with very closed patterns from pH 2 to pH 10, except in pH 11 with different pattern and in pH 12 with instant degradation. 1-NA was stable in all tested matrix at designed temperature. 1-NIC was turned out unstable with instant degradation in plasma, urine, and ACN extracts of plasma and urine, but stable in ACN. The degradation product of 1-NITC and 1-NIC in universal buffer was confirmed 1-NA. The degradation compound in plasma, urine, and ACN extracts of plasma and urine, was not identified. The developed HPLC assay was used in a preliminary pharmacokinetic study to analyze plasma and urine samples following the i.v. administration of 25 mg/kg 1-NITC to female Spraque-Dawley rats. 1-NITC and 1-NA were detected and quantified in rat plasma and urine, respectively. Based on noncompartmental analysis by WinNonLin, 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.

Acknowledgements

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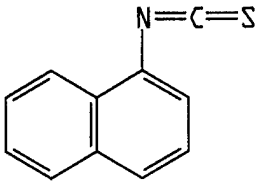
Fig. 1. The chemical structures of 1-NITC, 1-NA, 1-NIC, and NE



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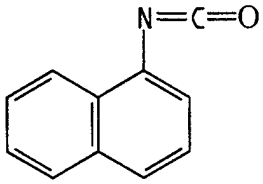

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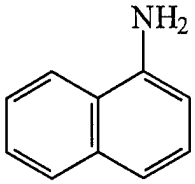

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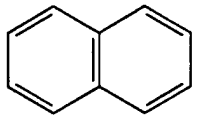
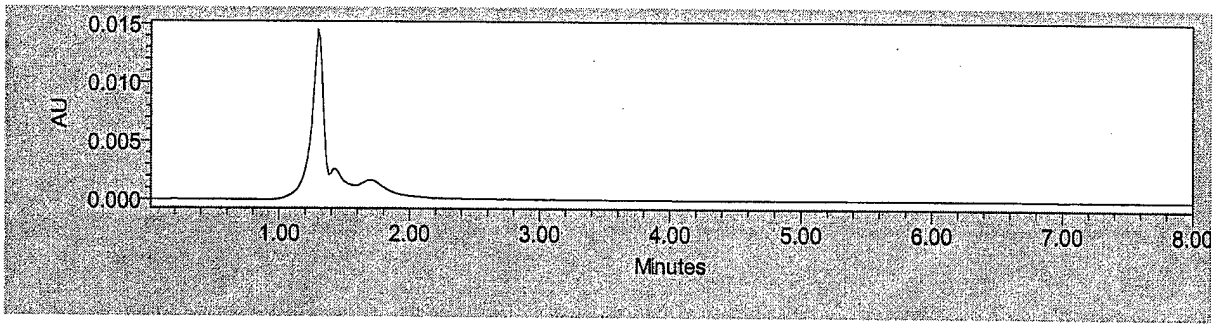


NE

Fig. 2. Typical chromatographs for rat plasma samples obtained from the analysis of (a) blank plasma. (b) ACN spiked with 1-NITC (200 ng/ml), 1-NA (200 ng/ml), 1-NIC (200 ng/ml), and NE (10 μg/ml). (c) blank plasma spiked with 1-NITC (200 ng/ml), 1-NA (200 ng/ml), 1-NIC (200 ng/ml), and NE (10 μg/ml), and followed protein precipitation with ACN. (d) blank plasma spiked with 1-NITC (200 ng/ml), 1-NA (200 ng/ml), and NE (10 μg/ml), followed protein precipitation with ACN, and 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 (shift with 5.4, 5.6 or 5.9 min in different matrix).

(2a)



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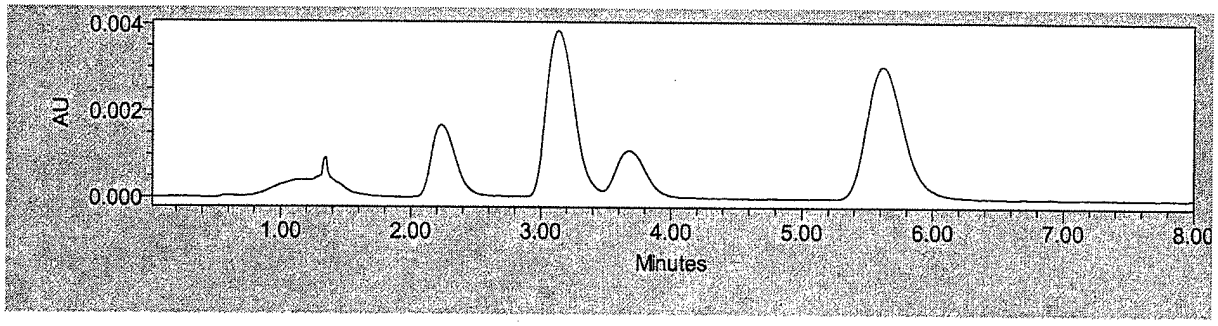

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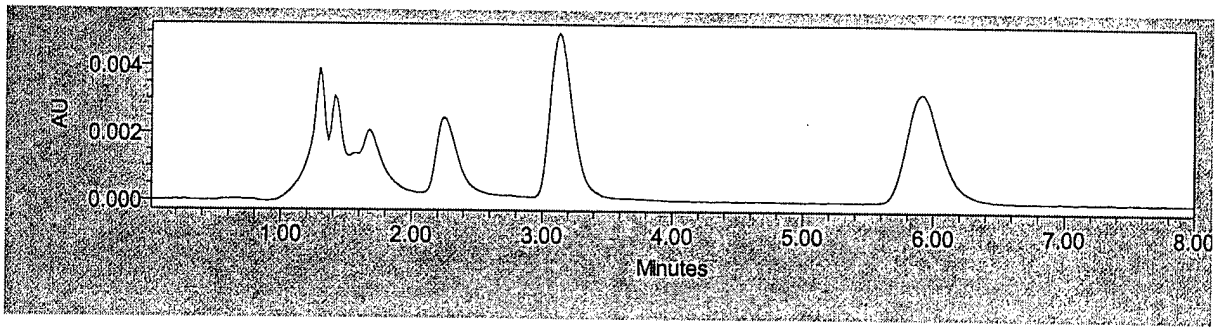
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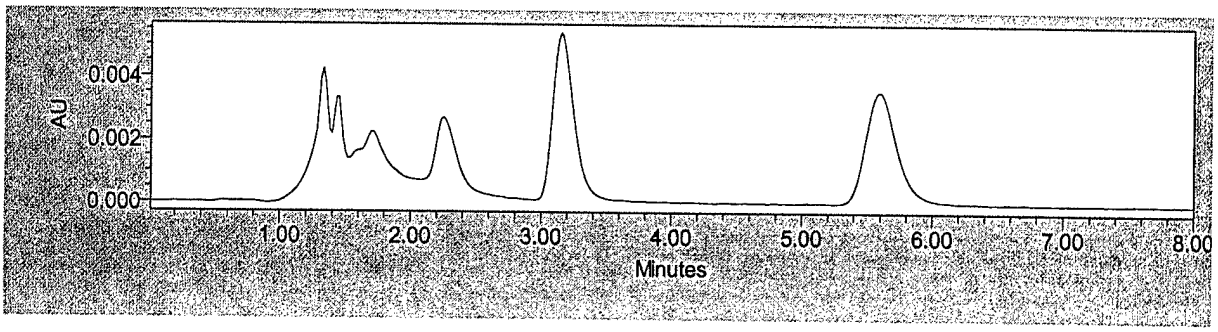
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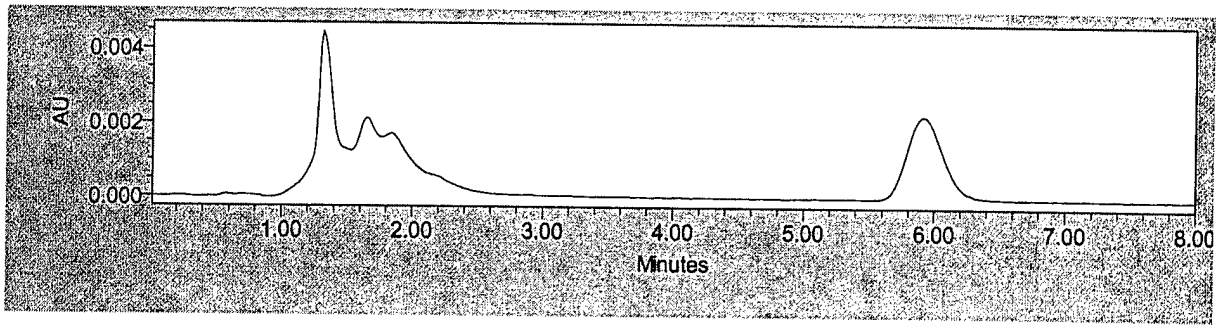
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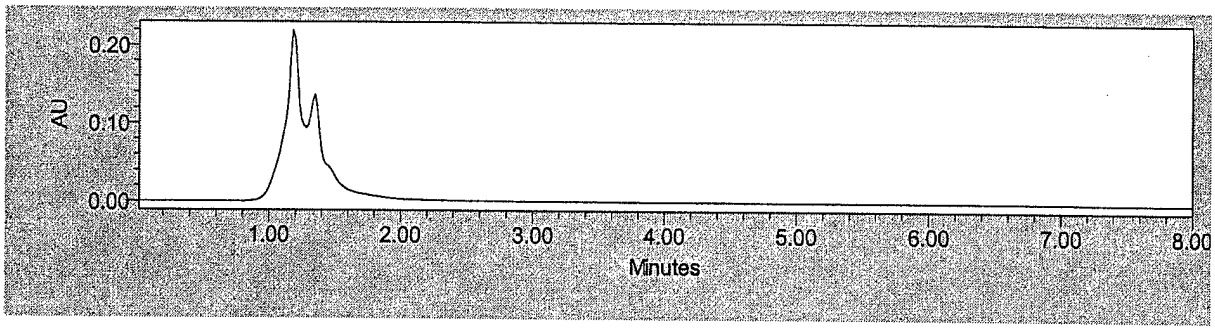
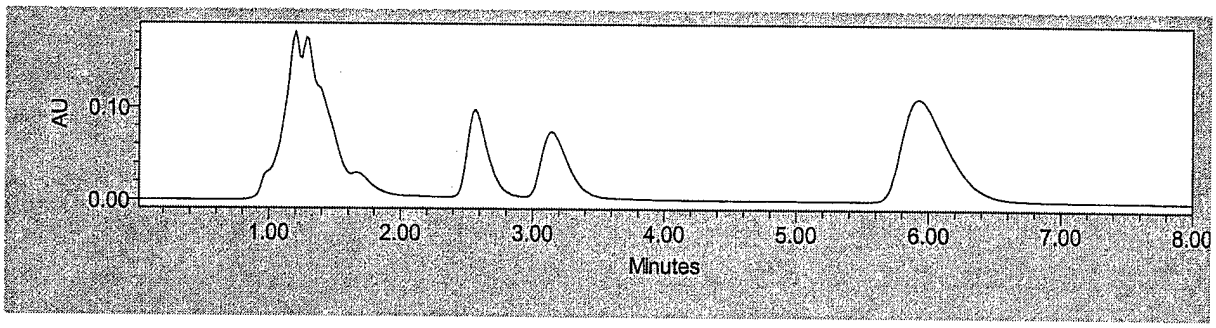
Fig. 3. Typical chromatographs for rat urine samples obtained from the analysis of (a) blank urine. (b) blank urine spiked with 1-NITC (10 μg/ml), 1-NA (10 μg/ml), 1-NIC (10 μg/ml), and NE (40 μg/ml), followed by extraction with ACN. (c) blank urine spiked with 1-NITC (10 μg/ml), 1-NA (10 μg/ml), and NE (40 μg/ml), following extraction with ACN, and the supernatant spiked with 1-NIC (10 μg/ml). (d) 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 (shift with 2.2 and 2.6 min), NE (3.2 min), and 1-NITC (6.0 min).

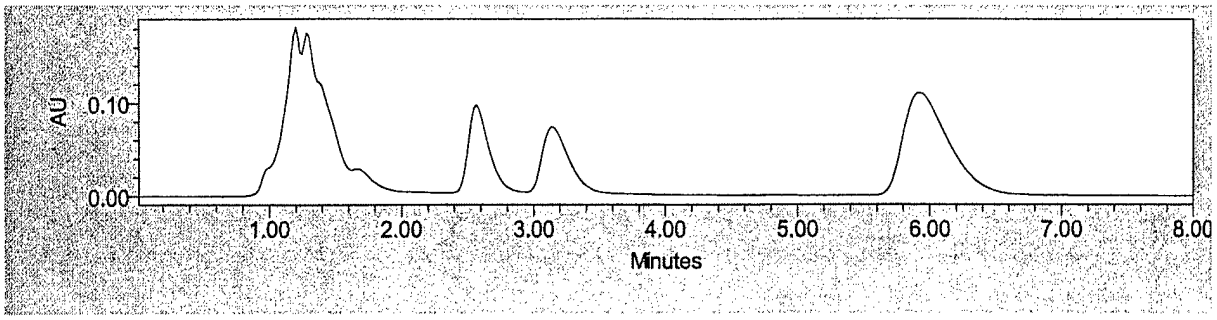
(3a)



(3b)



(3c)


AU
0.10
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Minutes

(3d)

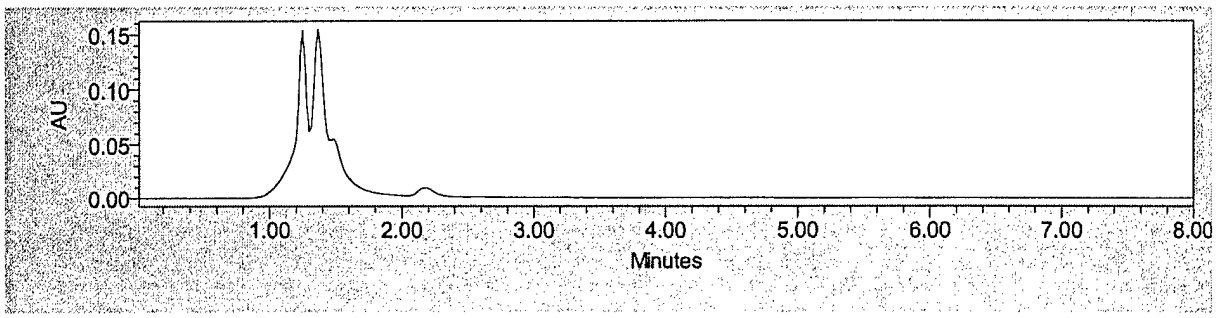
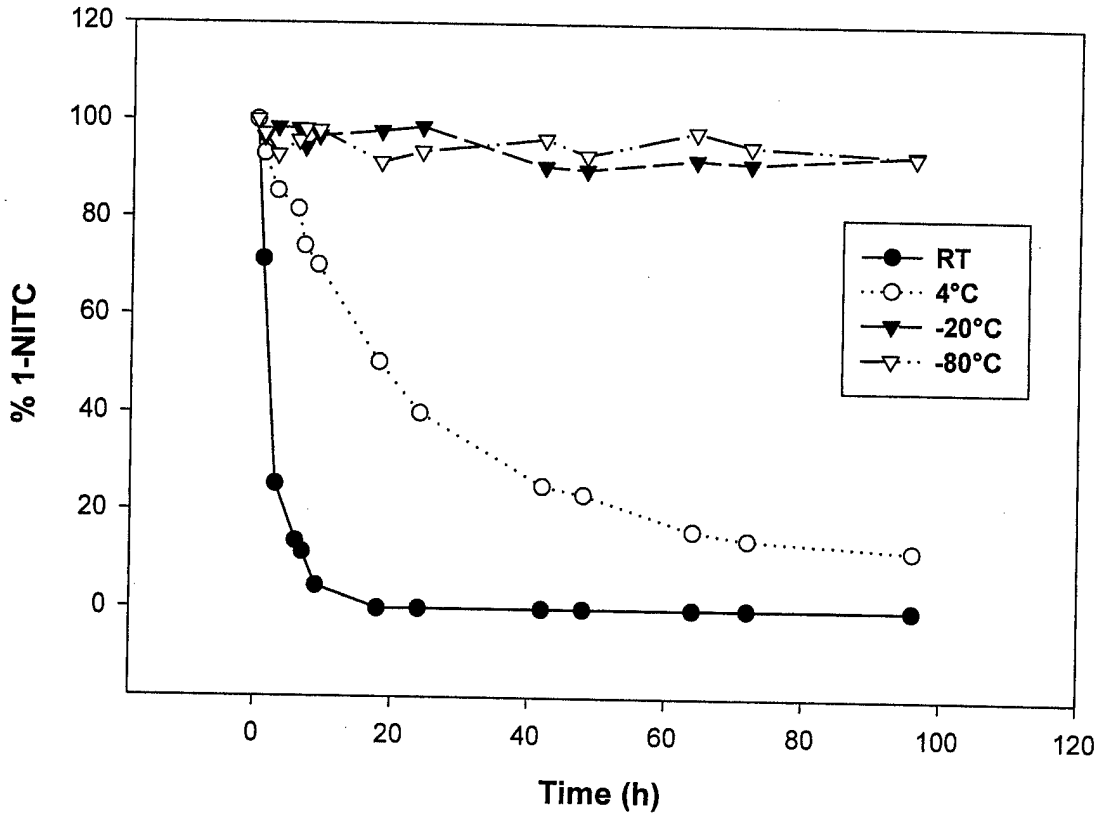
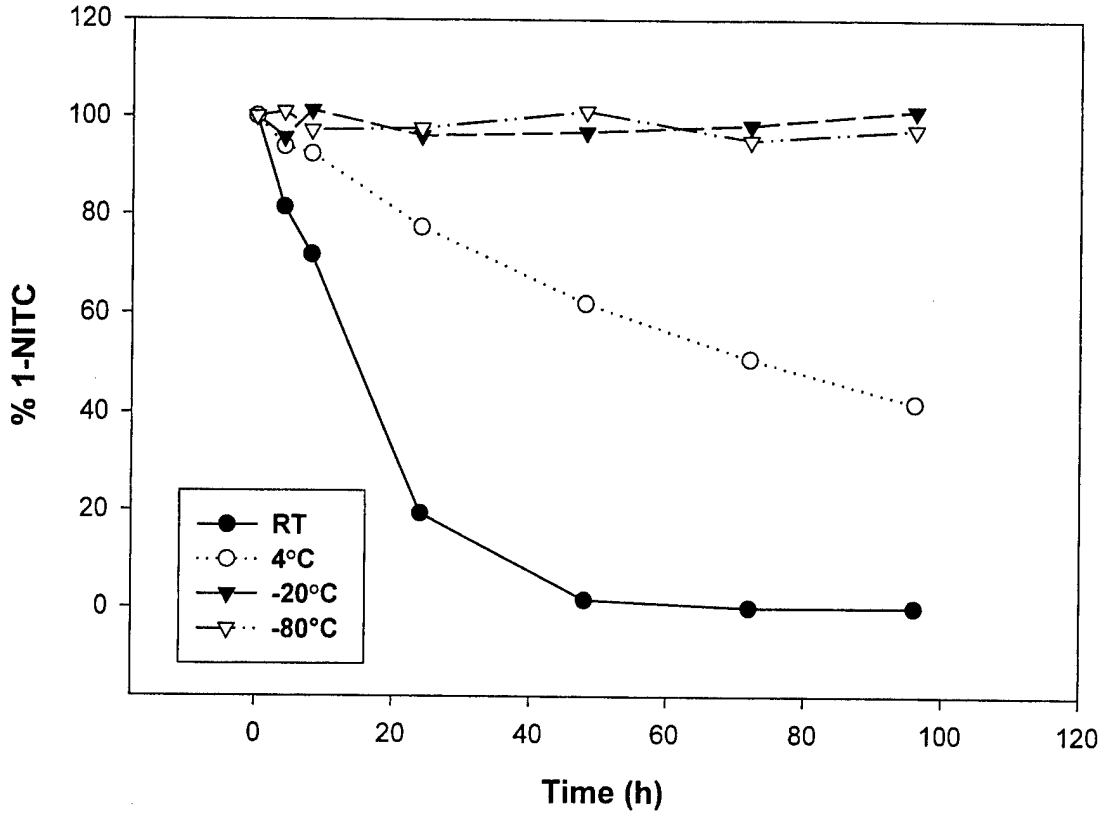

AU
0.15
0.10
0.05
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Minutes

Fig. 4. The stability of 1-NITC, 1-NA, and 1-NIC in rat plasma, urine, ACN extract of plasma and urine, ACN, and universal buffer at RT, 4°C, -20°C, and -80°C over 96 h.

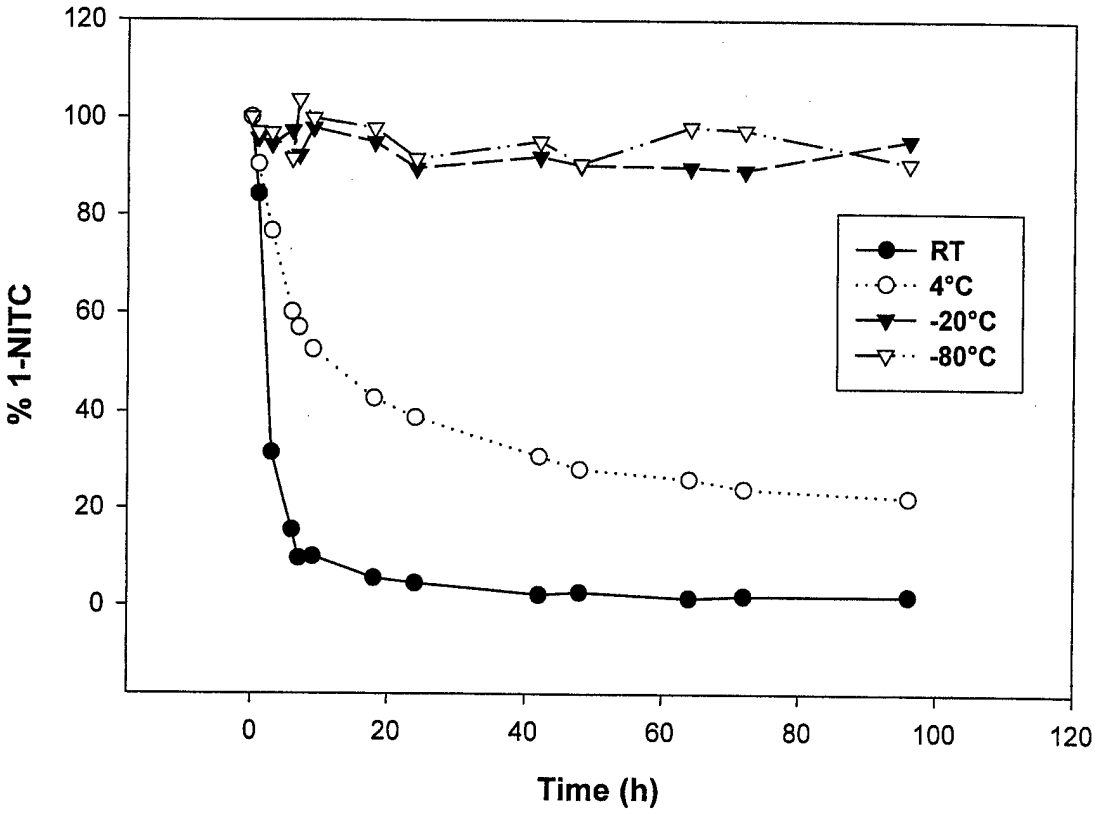
(4a) The stability of 1-NITC in rat plasma at RT, 4°C, -20°C, and -80°C at 0, 1, 3, 6, 7, 9, 18, 24, 42, 48, 64, 72, and 96 h



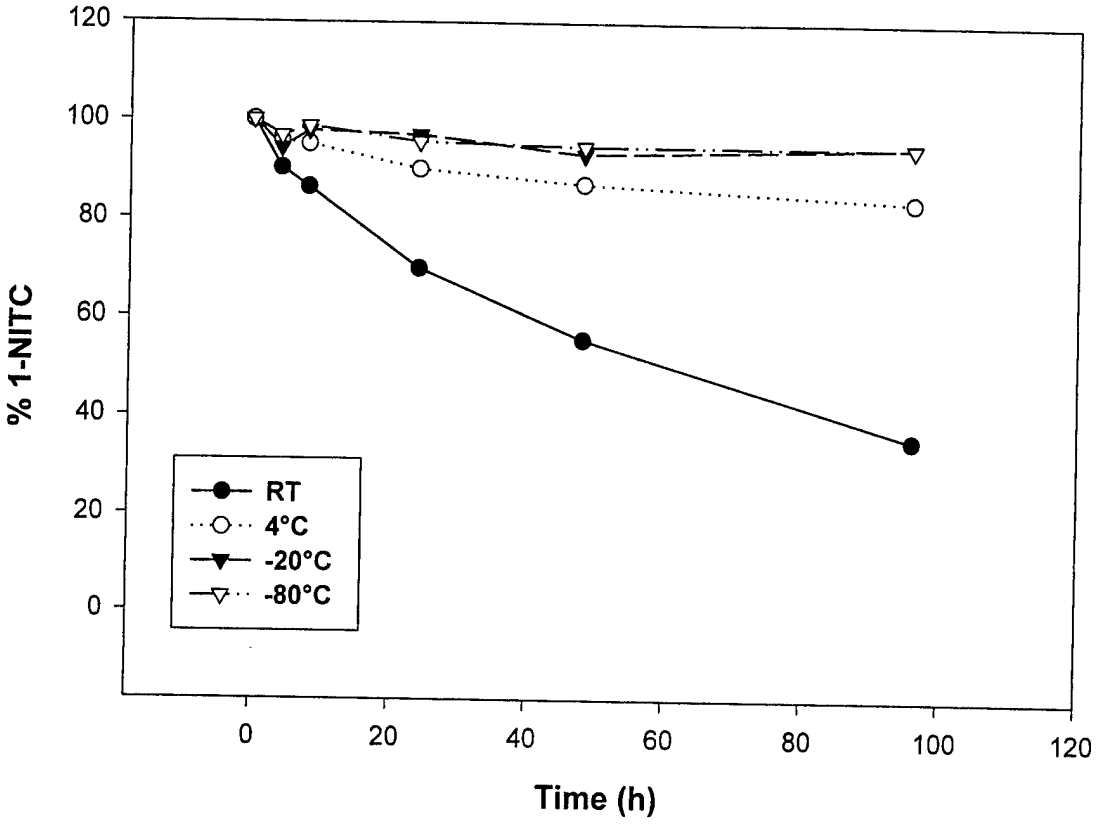
(4b) The stability of 1-NITC in ACN extracts of rat plasma at RT, 4°C, -20°C, and -80°C at 0, 4, 8, 24, 48, 72, and 96 h



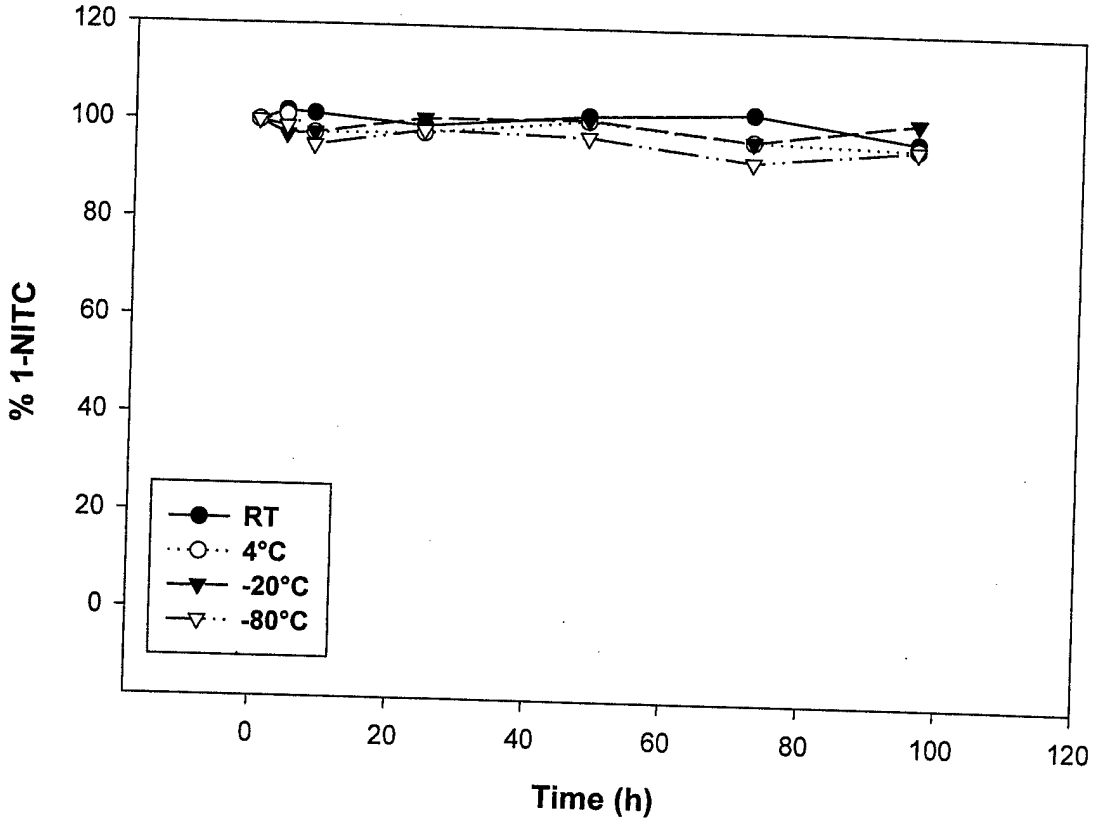
(4c) The stability of 1-NITC in rat urine at RT, 4°C, -20°C, and -80°C at 0, 1, 3, 6, 7, 9, 18, 24, 42, 48, 64, 72, and 96 h



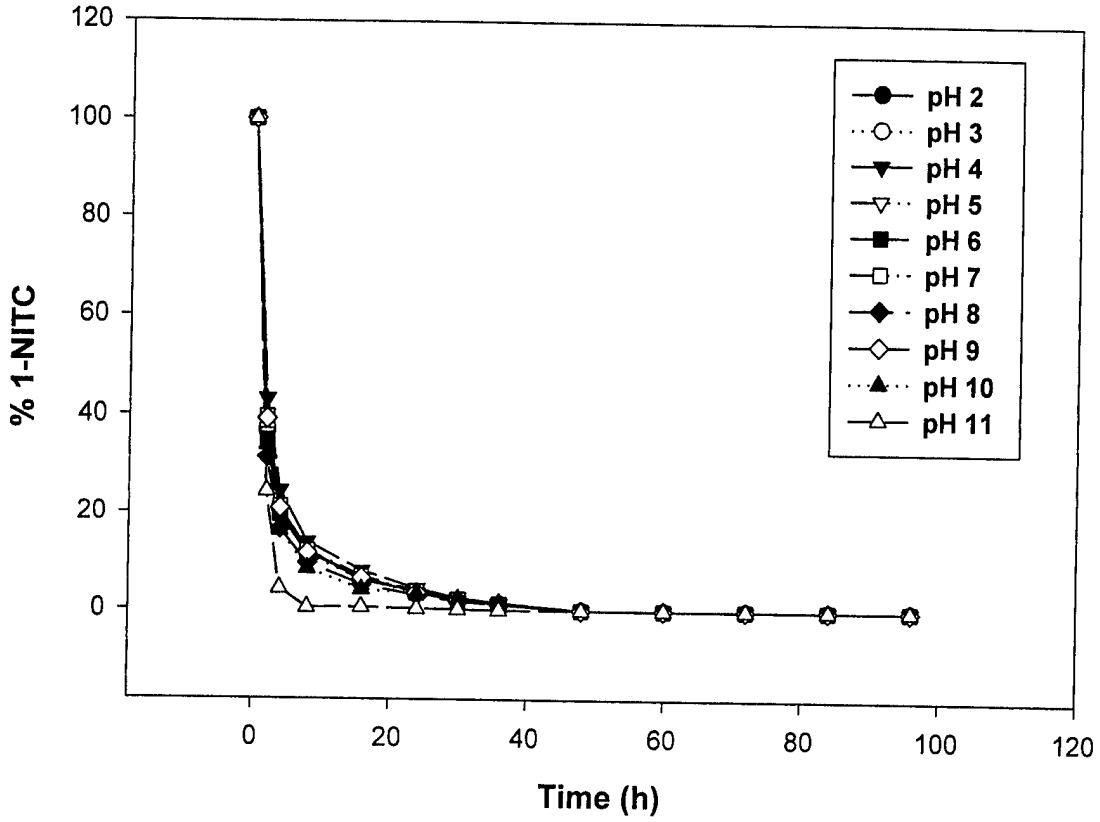
(4d) The stability of 1-NITC in ACN extracts of rat urine at RT, 4°C, -20°C, and -80°C at 0, 4, 8, 24, 48, 72, and 96 h



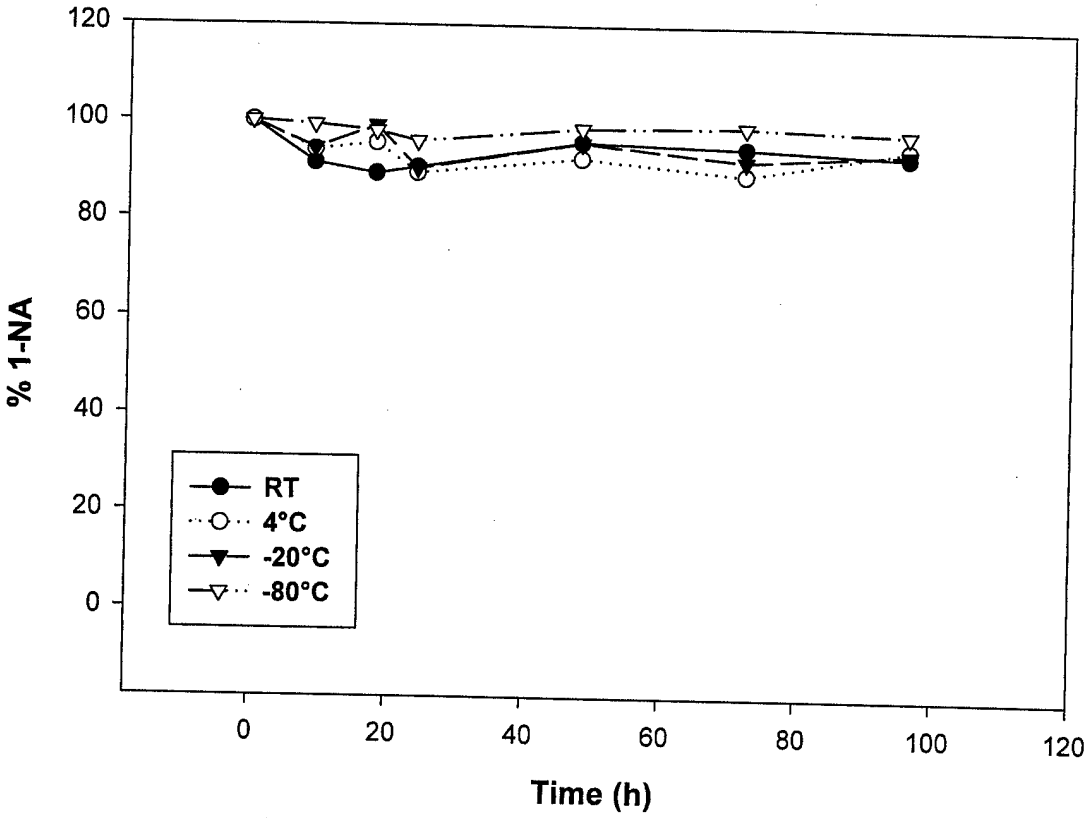
(4e) The stability of 1-NITC in ACN at RT, 4°C, -20°C, and -80°C at 0, 4, 8, 24, 48, 72, and 96 h



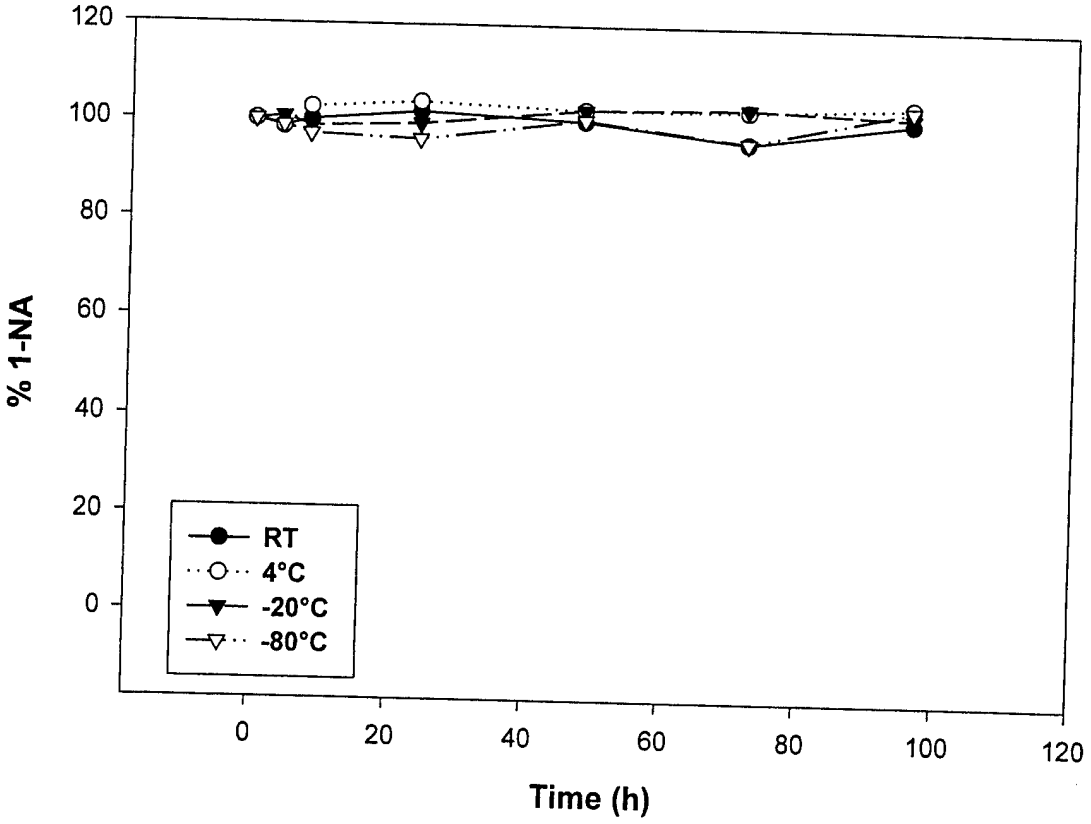
(4f) The stability of 1-NITC in universal buffer pH 2-12 at RT at 0, 2, 4, 8, 16, 24, 30, 36, 48, 60, 72, 84, and 96 h



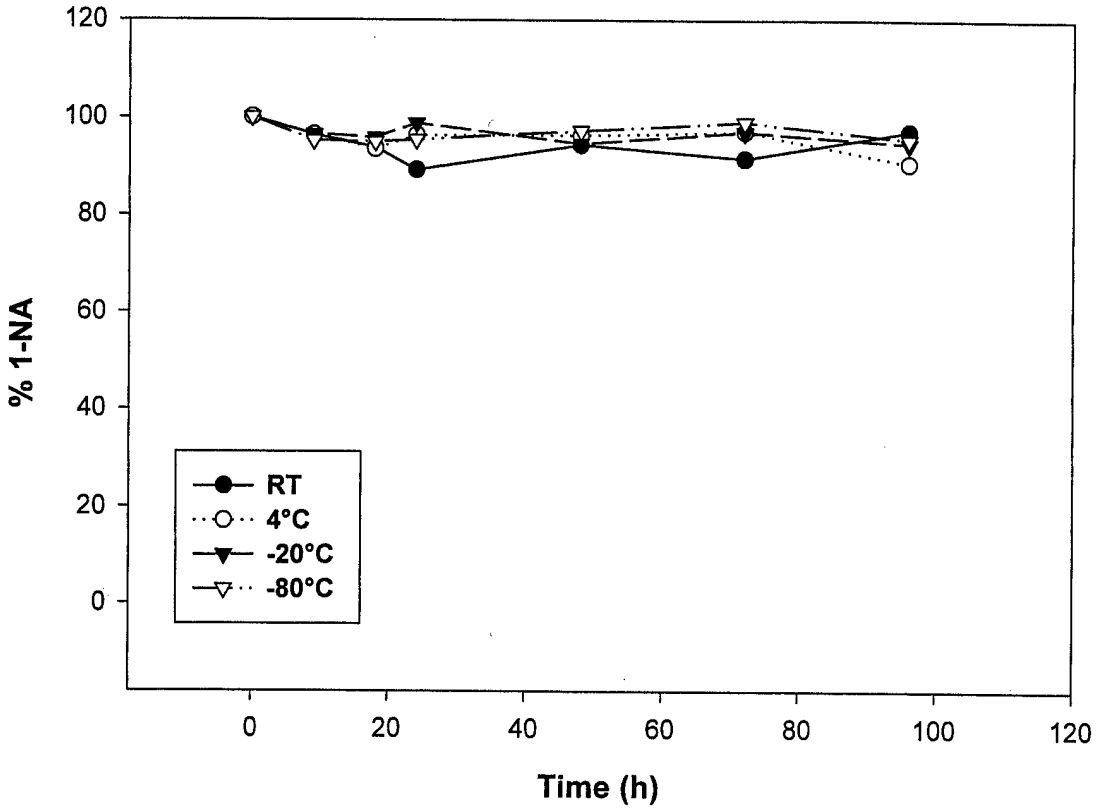
(4g) The stability of 1-NA in rat plasma at RT, 4°C, -20°C, and -80°C at 0, 9, 18, 24, 48, 72, and 96 h



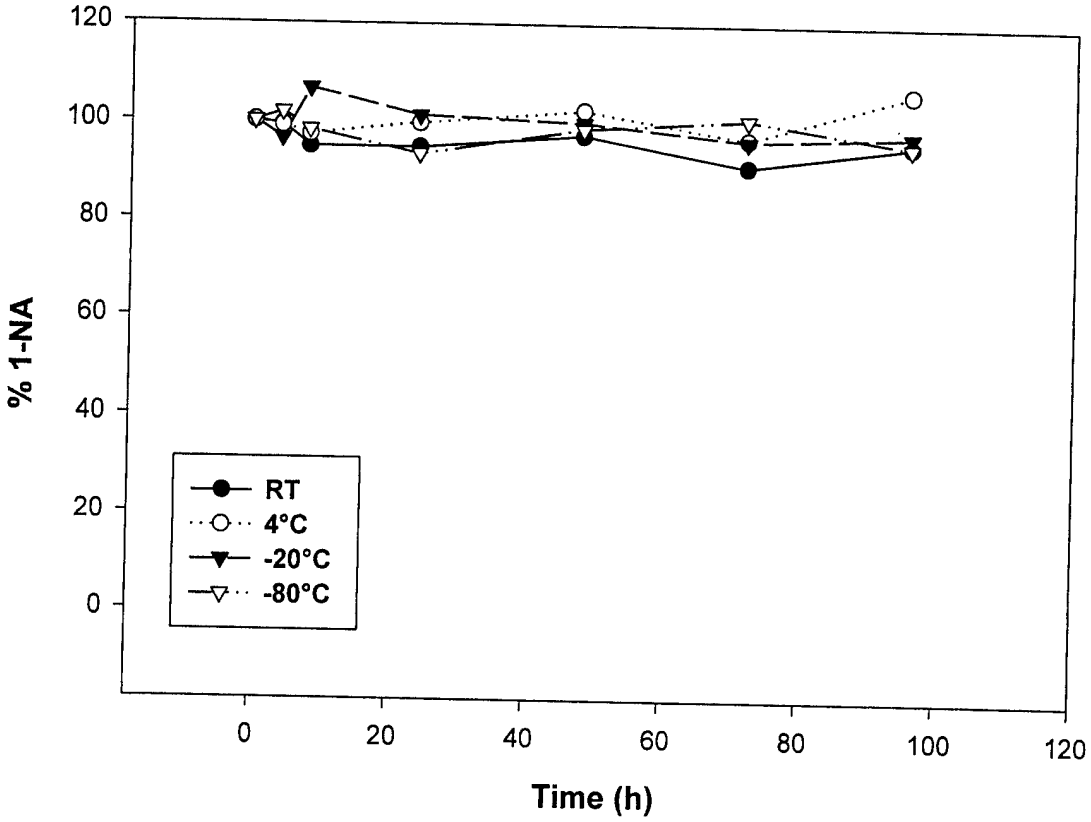
(4h) The stability of 1-NA in ACN extracts of rat plasma at RT, 4°C, -20°C, and -80°C at 0, 9, 18, 24, 48, 72, and 96 h



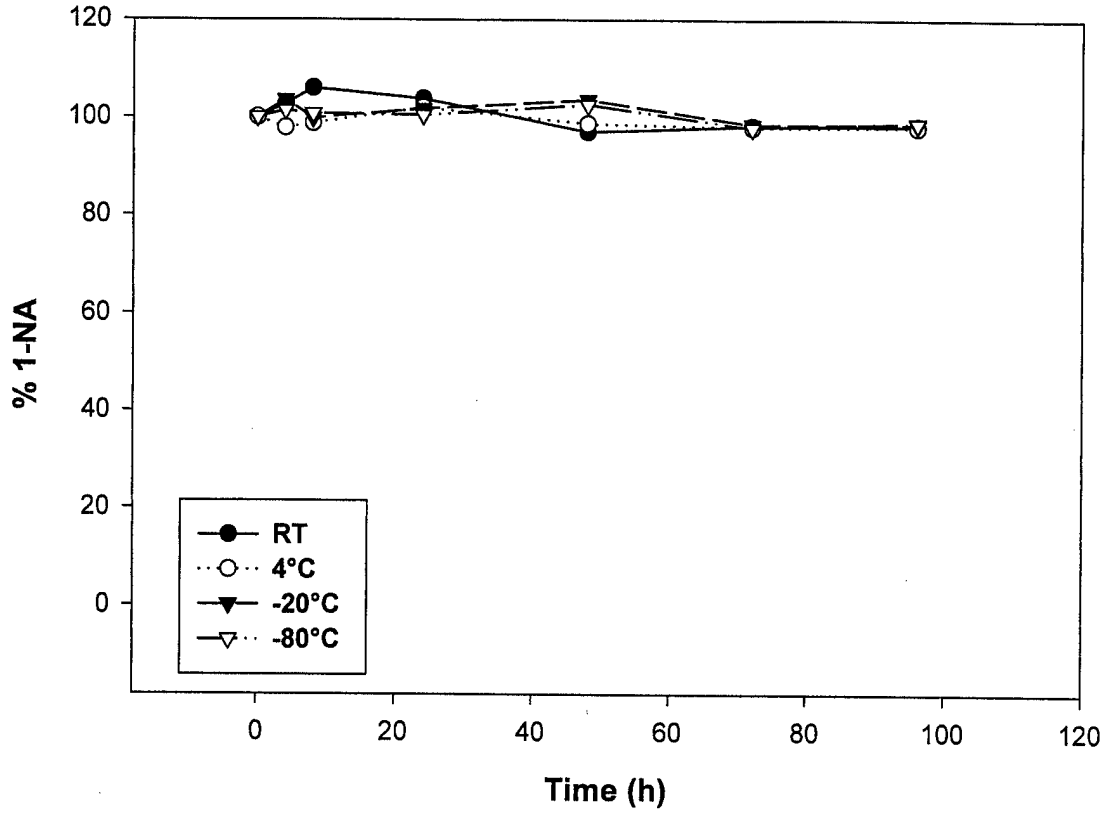
(4i) The stability of 1-NA in rat urine at RT, 4°C, -20°C, and -80°C at 0, 9, 18, 24, 48, 72, and 96 h



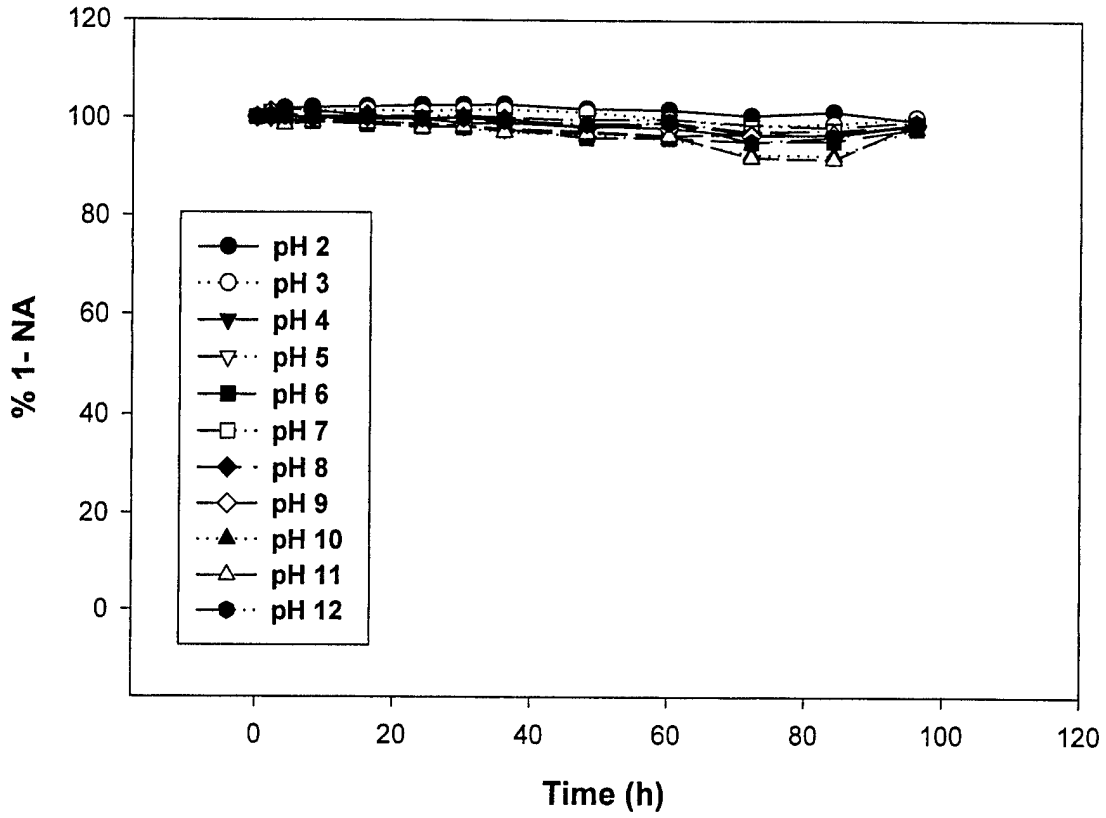
(4j) The stability of 1-NA in ACN extracts of rat urine at RT, 4°C, -20°C, and -80°C at 0, 4, 8, 24, 48, 72, and 96 h



(4k) 1-NA in ACN at RT, 4°C, -20°C, and -80°C at 0, 4, 8, 24, 48, 72, and 96 h



(4l) The stability of 1-NA in universal buffer pH 2-12 at RT at 0, 2, 4, 8, 16, 24, 30, 36, 48, 60, 72, 84, and 96 h



(4m) 1-NIC in ACN at RT, 4°C, -20°C, and -80°C at 0, 4, 8, 24, 48, 72, and 96 h

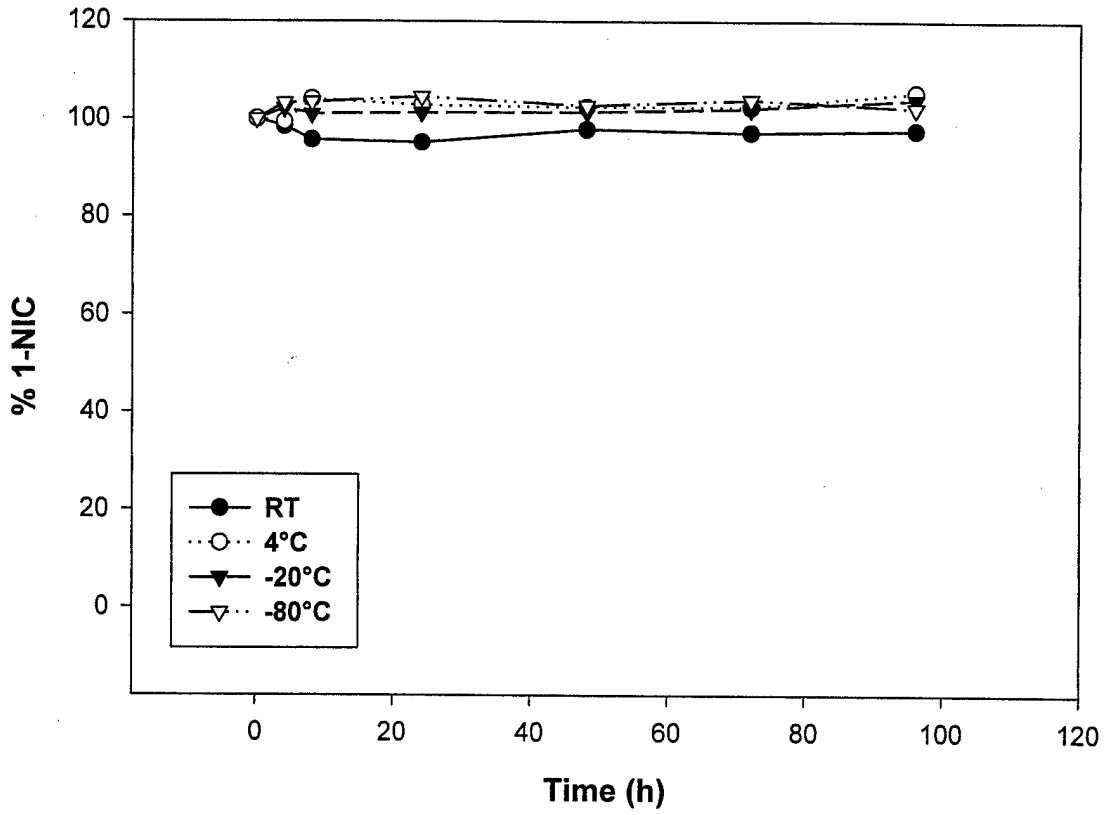


Fig. 5. The concentration 1-NITC vs. time in rat plasma after an i.v. bolus of 25 mg/kg dose

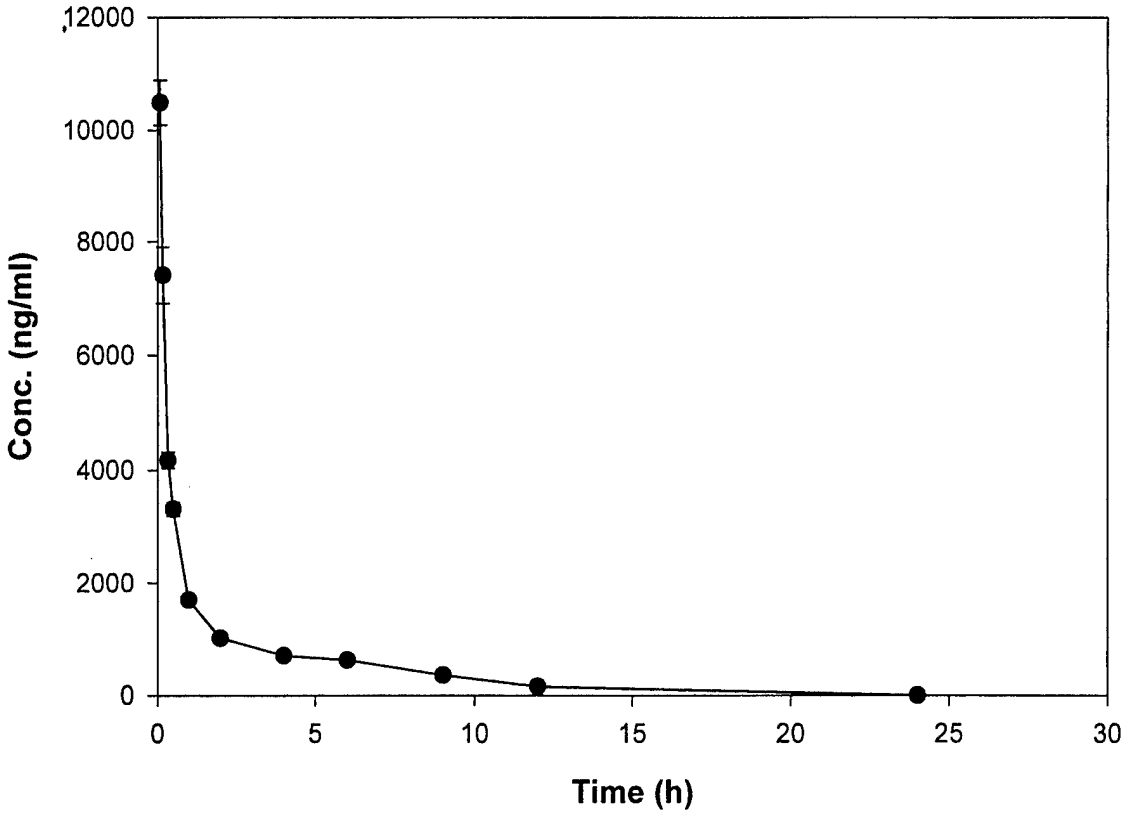


Table 1. The lower limit of quantitation (LLQ) of 1-NITC, 1-NA, and 1-NIC in rat plasma (PLM), urine (URN), and ACN

Compounds	**LLQ in PLM (ng/ml)**	**LLQ in URN (ng/ml)**	**LLQ in ACN (ng/ml)**
1-NITC	10	30	10
1-NA	30	100	30
1-NIC	ND[a]	ND	30

[a] ND: not detected in blank plasma and urine samples.

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

	QCs (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 HPLC validation of 1-NA in rat urine

	QCs (ng/ml)	Accuracy (%)	Precision (%)	Recovery (%)
Within-Day	100	106	98.7	102
	5000	98.2	99.4	107
	50000	100	98.4	110
Between-Day	100	105	97.4	95.4
	5000	96.3	97.7	104
	50000	100	99.3	107

Table 4. The concentration of 1-NITC in rat plasma with 25 mg/kg i.v. bolus administration

Plasma Sample ID No.	Time	Conc. (ng/ml)
PLM 1	5 min	10490 ± 400
PLM 2	10 min	7405 ± 498
PLM 3	20 min	4172 ± 140
PLM 4	30 min	3312 ± 118
PLM 5	1 h	1692 ± 77
PLM 6	2 h	1016 ± 48
PLM 7	4 h	702 ± 36
PLM 8	6 h	620 ± 29
PLM 9	9 h	351 ± 15
PLM 10	12 h	150 ± 12
PLM 11	24 h	0

Table 5. The concentration of 1-NA in rat urine with 25 mg/kg i.v. bolus administration

Urine Sample ID No.	Time Interval	Vol. (ml)	Conc. (μg/ml)	Amount (μg)
URN 1	0-2 h	8.2	0.42 ± 0.04	3.44 ± 0.33
URN 2	2-4 h	3.8	2.02 ± 0.23	7.68 ± 0.87
URN 3	4-6 h	1.5	2.64 ± 0.35	3.96 ± 0.52
URN 4	6-9 h	1.5	2.17 ± 0.25	3.26 ± 0.37
URN 5	9-24 h	30	0	0
URN 6	24-25 h	3.2	0	0
Total Amount (μg)				18.34 ± 2.09

Cytotoxicity of Dietary Organic Isothiocyanates in Human Breast Cancer Cells

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Purpose. Organic isothiocyanates (ITCs) (mustard oils) are present in cruciferous vegetables and known to have cancer chemopreventative properties due to their inhibition of carcinogen metabolic activation. The purpose of this investigation was to examine the cytotoxicity of 1-naphthyl isothiocyanate (NITC), benzyl isothiocyanate (BITC) and β-phenylethyl isothiocyanate (PEITC) in a multidrug-resistant human breast cancer cell line, MCF-7/ADR. **Methods.** The cytotoxicity of PEITC, BITC, NITC, daunomycin (DNM) and vinblastine (VBL) were evaluated following the same exposure times at concentrations varying from 0.1 to 50 μM. The compounds were added to the cultured cells in 96-well plates for an exposure period of 48 hours and cell growth was determined by a sulforhodamine B assay. DNM and VBL were used to standardize the conditions of the assay, and to obtain IC_{50} values for comparative purposes. **Results**. Based on preliminary studies with DNM, VBL and PEITC, we found that IC_{50}'s decreased with increasing exposure times, and chose exposure times of 48 hours for our studies. The IC_{50} values for DNM and VBL were 1.59 ± 0.093 μM (mean ± SE) and 0.106 ± 0.004 μM, respectively. PEITC and BITC inhibited cell growth with IC_{50}'s of 6.62 μM ± 0.185 μM and 5.90 ± 0.129 μM. NITC demonstrated no cytotoxicity at concentrations of 10 μM or less. **Conclusions**. BITC and PEITC, which are present in cruciferous vegetables in mg quantities, have a cytotoxic effect comparable to the anticancer drugs, DNM and VBL, in a multidrug resistant human breast cancer cell line. NITC exhibited no cytotoxicity at similar concentrations.

Support provided by grants from NY Dept. of Health, the Susan G. Komen Foundation and the US Army MRMC (IDEA grant).

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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.

HPLC Assay and Stability of Phenethyl Isothiocyanate

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Purpose. Phenethyl isothiocyanate (PEITC) is a component present in cruciferous vegetables that has cancer chemopreventive properties. Our objectives were to develop an HPLC assay for PEITC and its mercapturic acid conjugate, and to determine the pH-and temperature-dependent stability of PEITC. The assay was used to evaluate PEITC and its mercapturic acid conjugate in urine samples from subjects following watercress ingestion. **Methods.** The HPLC mobile phase for PEITC and its metabolite were methanol:water (60:40, v/v) and acetonitrile:10mM phosphate buffer pH 3.0 (30:70, v/v), respectively. Propiophenone was used as the internal standard. PEITC mercapturic acid was synthesized based on the method of Adesida, et al., 1996 and Brusewitz, et al., 1977. The limit of quantitation of PEITC and its mercapturic acid conjugate was 4 μM . Stability of PEITC was studied in universal buffers of citrate-phosphate-borate/HCl at pH values of 3.0, 5.0, 7.4, 8.4, 9.4 and 10.4. All samples were studied following incubation at RT and at refrigerated temperatures. Four healthy subjects ingested 100g of fresh watercress and urine samples were collected over time and analyzed for PEITC and PEITC mercapturic acid conjugate. **Results.** PEITC degrades with first order kinetics in buffer solutions. The half-life of PEITC in a buffer of pH 7.4 at RT is 56.1 hours and at 4 °C is 108.1 hours. Urine samples obtained after watercress ingestion contained only the PEITC mercapturic acid conjugate, with greatest amounts appearing between 2-4 hours. The minimum value for bioavailability, based on urinary recovery of the mercapturic acid conjugate of PEITC, was variable with a geometric mean of 44.5% and a median value of 52.5%. **Conclusions.** An HPLC assay for PEITC and its mercapturic acid conjugate has been developed. The PEITC and its metabolite are stable under the sample collection and assay conditions.

Supported by grants from the Susan G. Komen Foundation and the U.S. Army Breast Cancer Research Program, Contract DAMD17-00-1-0376.

American Assoc. of Pharmaceutical Scientist Annual Meeting, Nov. 2002

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

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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 ($r^2 = 0.77$ 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.

Era of Hope Meeting, Sept 2002

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