Modulation of human cytochrome P450 3A4 (CYP3A4) and P-glycoprotein (P-gp) in Caco-2 cell monolayers by selected commercial-source milk thistle and goldenseal products[1]

Jason W. Budzinski, Vance L. Trudeau, Cathy E. Drouin, Mitra Panahi, J. Thor Arnason, and Brian C. Foster

Abstract: In this study, we used an in vitro Caco-2 cell monolayer model to evaluate aqueous extracts of commercial-source goldenseal (*Hydrastis canadensis*) and milk thistle (*Silybum marianum*) capsule formulations, their marker phytochemicals (berberine and silibinin, respectively), as well as dillapiol, vinblastine, and the HIV protease inhibitor saquinavir for their ability to modulate *CYP3A4* and *ABCB1* expression after short-term exposure (48 h). Both upregulation and downregulation of *CYP3A4* expression was observed with extracts of varying concentrations of the two natural health products (NHPs). *CYP3A4* was highly responsive in our system, showing a strong dose-dependent modulation by the *CYP3A4* inhibitor dillapiol (upregulation) and the milk thistle flavonolignan silibinin (downregulation). *ABCB1* was largely unresponsive in this cellular model and appears to be of little value as a biomarker under our experimental conditions. Therefore, the modulation of *CYP3A4* gene expression can serve as an important marker for the in vitro assessment of NHP–drug interactions.

Key words: ABCB1, Caco-2, CYP3A4, gene expression, goldenseal, HIV, milk thistle, P-glycoprotein, natural health product.

Résumé : Dans la présente étude, on a examiné divers extraits aqueux de formulations en capsule de chardon Marie (*Silybum marianum*) et d'hydraste du Canada (*Hydrastis canadensis*) de source commerciale, leurs constituants phytochimiques (berbérine et silibinine respectivement) dillapiol, vinblastine, et l'inhibiteur de la protéase du HIV saquinavir afin d'évaluer leur capacité à moduler l'expression de *CYP3A4* et *ABCB1* dans des monocouches de cellules Caco-2 après une exposition de courte durée (48 heures). Les extraits aqueux de diverses concentrations des deux produits de santé naturels (PSN) ont modulé dans une large mesure l'expression du *CYP3A4*, puisqu'une augmentation et une diminution ont été observées. En règle générale, *CYP3A4* a fortement réagi dans notre système, tel qu'indiqué par une modulation dose-dépendante importante par l'inhibiteur de *CYP3A4* dillapiol (augmentation) et la flavolignane silibinine du chardon Marie (diminution). *ABCB1* a faiblement réagi dans ce modèle cellulaire et offre peu d'intérêt comme biomarqueur dans nos conditions expérimentales. Ainsi, la modulation de l'expression du gène *CYP3A4* pourrait être un marqueur important pour l'évaluation in vitro des interactions PSN-médicaments.

Mots-clés : ABCB1, Caco-2, CYP3A4, expression génique, hydraste du Canada, HIV, chardon Marie, P-glycoprotéine, produit de santé naturel.

[Traduit par la Rédaction]

Introduction

In the past decade, there has been a dramatic increase in the use of natural health products (NHPs), particularly herbals, which are often combined with conventional drug therapies. It is evident that NHPs are frequently sought by patients as part of a therapeutic regime of complementary or alternative medicine (CAM). For example, CAM use among North American patients who are HIV-positive may be as high as 70% (Mills et al. 2005). Two NHPs commonly

Received 30 November 2006. Published on the NRC Research Press Web site at cjpp.nrc.ca on 11 October 2007.

J.W. Budzinski, V.L. Trudeau, and J.T. Arnason.[2] Centre for Research in Biopharmaceuticals and Biotechnology, University of Ottawa, Ottawa, ON K1N 6N5, Canada; Ottawa-Carleton Institute of Biology, University of Ottawa, Ottawa, ON K1N 6N5, Canada.
C.E. Drouin and M. Panahi. Centre for Research in Biopharmaceuticals and Biotechnology, University of Ottawa, Ottawa, ON K1N 6N5, Canada.
B.C. Foster. Centre for Research in Biopharmaceuticals and Biotechnology, University of Ottawa, Ottawa, ON K1N 6N5, Canada; Office of Science, Therapeutic Products Directorate, Health Canada, Ottawa, ON K1A 1B6, Canada.

[1]This article is one of a selection of papers published in this special issue (part 1 of 2) on the Safety and Efficacy of Natural Health Products.

[2]Corresponding author (e-mail: john.arnason@uottawa.ca).

 doi:10.1139/Y07-091

Table 1. Summary of the commercial-source goldenseal and milk thistle capsules used for *CYP3A4* and *ABCB1* expression studies, including individual product label information.

			Product label information				
NRP No.	Company location (origin)	Unit mass; medicinal ingredient source; amount in product	Dosage	Nonmedicinal ingredients	Quantity dried equivalent dose per day	Product cost (CDN$)	Cost/ capsule (CDN$)
Goldenseal (*Hydrastis canadensis*)							
121	British Columbia, Canada	450 mg; root powder; 90 capsules	1–3 capsules daily with meals	None listed	450–1350 mg of root powder	19.71	0.22
17	Utah, USA	250 mg of root extract standardized to 10% alkaloids; 65 mg of root; 60 capsules	1–2 capsules daily with meals	Gelatin (capsule), magnesium stearate	250–500 mg of root extract (25–50 mg of alkaloids) plus 65–130 mg of root powder	34.99	0.58
Milk thistle (*Silybum marianum*)							
126	Ontario, Canada	250 mg of extract standardized to 80% silymarins; source not listed; 60 capsules	1–2 capsules 3× daily with meals	Rice flour, dicalcium phosphate, silica, magnesium stearate	750–1500 mg of herb extract (600–1200 mg silymarins)	16.09	0.27
18	Utah, USA	175 mg of seed extract standardized to 140 mg (80%) silymarin; source not listed; 120 capsules	1 capsule 1–2× daily with meals	Cellulose, gelatin (capsule), silica, magnesium stearate	175–350 mg of seed extract (120–240 mg silymarins)	45.99	0.38

Note: NRP No., designated in-house natural research product number.

utilized by HIV-positive patients in CAM therapy are goldenseal (*Hydrastis canadensis*) (GS) for its purported antibacterial, antiviral, and immunostimulatory properties, and milk thistle (*Silybum marianum*) (MT), taken for co-infections with hepatitis for its purported ability to promote liver health (Lee et al. 2006). An increasing body of research suggests, however, that many NHPs and their constituents can alter the drug disposition (i.e., the resorption, metabolism, and elimination) of antiretroviral drugs, which may or may not lead to the development of clinically significant toxic adverse reactions and (or) a decrease in therapy effectiveness (Foster et al. 2001, 2005; Gurley et al. 2004, 2005; Lee et al. 2006; Mouly et al. 2004; Patel et al. 2004; Piscitelli et al. 2002*a*, 2002*b*; Sandhu et al. 2003).

In general, NHPs have biologically active constituents that may exert significant pharmacological activities capable of producing both potent therapeutic effects and adverse reactions, thereby affecting human drug disposition processes (Foster et al. 2005). Although drug disposition is mediated by numerous detoxification mechanisms acting simultaneously, each mechanism potentially affected by NHP–drug interactions is part of a dynamic interplay in the in vivo state and NHP constituents may be substrates for more than 1 metabolic enzyme or transporter (Ameer and Weintraub 1997). In this regard, 2 key drug disposition processes commonly investigated for assessing NHP–drug interactions are the metabolism of substrates by the phase I drug-metabolizing enzyme cytochrome P450 3A4 (CYP3A4), and the transport of substrates by the ATP-dependent membrane-bound glycoprotein P-glycoprotein (P-gp) (170 kDa). NHP constituents can inhibit CYP3A4 thereby altering the presystemic metabolism of orally coadministered drugs because CYP3A4 has broad substrate specificity, mediates many interactions with xenobiotics, and oxidizes approximately 50% of orally administered drugs (Thummel and Wilkinson 1998). Many NHPs or drugs that inhibit CYP3A4 can also influence the P-gp efflux of concomitantly administered substrates, since both CYP3A4 and P-gp have numerous overlapping substrate specificities (Cummins et al. 2001, 2002; Kivistö et al. 2004; Mouly et al. 2004). In malignant cells, overexpressed P-gp can lead to the development of multi-drug resistance in chemotherapy, and early work investigating P-gp has mainly related it to anticancer agents (Wandel et al. 1999). Both in vitro and in vivo studies have established that GS and its isoquinoline alkaloid berberine can inhibit CYP3A4 (Budzinski et al. 2000; Chatterjee and Franklin 2003) and P-gp (Stermitz et al. 2000) activities. The same has been found for MT and its flavonolignan silibinin with respect to CYP3A4 (Sridar et al. 2004; Venkataramanan et al. 2000) and P-gp (Maitrejean et al. 2000; Zhou et al. 2004) inhibition. In previous investigations (Budzinski et al. 2001) and other studies (Budzinski 2003), we demonstrated that MT and GS extracts significantly stimulated P-gp ATPase activity compared with the positive control and other tested NHPs. In this study, Caco-2 cell monolayers were treated with varying concentrations of aqueous extracts prepared from commercial-source GS and MT capsule products, along with berberine and silibinin (their respective marker phytochemicals), and vinblastine, dilla-

piol, and the HIV protease inhibitor saquinavir. Treatments consisted of a short-term exposure (48 h) and observed levels of *ABCB1* and *CYP3A4* expression in response to the treatments.

Materials and methods

Herbal product extracts

MT and GS products were purchased from local retail outlets (Ottawa, Ont.) and assigned unique natural research product (NRP) designation numbers; these products are summarized in Table 1. To achieve a biologically relevant test concentration, aqueous extracts of 4 selected herbal products were made on the basis of the calculated maximal daily dosage indicated on the label of each product. It was decided that a volume of 200 mL (i.e., a typical volume for a glass of water) was an appropriate volume for concentration calculations. Aqueous extracts of each product were made fresh with 200 mL ddH_2O as stock solutions that were 10× more concentrated than the maximal daily dosage. The appropriate amount of herbal product was weighed out from the contents of 3–5 capsules (combined in a small weigh-boat) and added to 20 mL of ddH_2O in a 50 mL Falcon centrifuge tube (note that the 10× stock solutions were scaled down from 200 mL to a volume of 20 mL). This suspension, consisting of various soluble plant components, such as phytochemicals, in solution, as well as insoluble fibrous plant material, was then vortexed for 1 min, sonicated for 1 min, vortexed again for 1 min, sonicated again for 1 min, and finally centrifuged at 500*g* for 10 min. The resultant supernatant was transferred to a different 50-millilitre tube and used to prepare a decade series of dilutions, resulting in 4 test concentrations for each herb. These herbal extracts were ultimately tested in the Caco-2 cell monolayers at 10% of the media volume at the following final test concentrations: 3.15 μg/mL to 3.15 mg/mL for GS-NRP 17; 6.75 μg/mL to 6.75 mg/mL for GS-NRP 121; 1.75 μg/mL to 1.75 mg/mL for MT-NRP 18; and 7.50 μg/mL to 7.50 mg/mL for MT-NRP 126.

Pure compounds

The phytochemical markers berberine and silibinin, for GS and MT, respectively (Harborne and Baxter 1993), were obtained from Sigma Aldrich (St. Louis, Mo.). The protease inhibitor saquinavir, an anti-HIV drug and substrate of P-gp and CYP3A4 (Mouly et al. 2004), was provided by Dr. Rolf van Heeswijk (the Ottawa Hospital, Ottawa, Ont.) in the form of a methanolic stock solution (0.165 mmol/L). The phytochemical dillapiol was used as an inhibitor of CYP3A4 (Budzinski et al. 2000) and was isolated from *Piper aduncum* as described in Bernard et al. (1995). Finally, the P-gp modulator vinblastine (Parasrampuria et al. 2001) was also obtained from Sigma Aldrich as vinblastine sulfate. All pure compounds were crystalline or powdered solids with the exception of dillapiol, which was an oily liquid. All pure compounds were maintained as 10 mmol/L stock solutions in 70% EtOH and were stored at –20 °C in the dark until needed. For specific Caco-2 cell treatments, the appropriate amount of pure compound was diluted directly into the media from the stock solution to yield the final test concentration.

HPLC analysis

GS and MT capsules used in this and concurrent studies (Budzinski 2003) were analyzed for levels of constituent marker compounds, namely, hydrastine and berberine for GS, and silibinin and the silymarin group for MT (Table 2). Crude capsule contents from each NRP (500 mg) were placed in 50-millilitre centrifuge tubes and extracted with analytical-grade solvents: 8 mL of 70% ethanol, 8 mL of methanol, or 8 mL of ddH_2O. Sample solutions were sonicated for 5 min, vortexed for 1 min, and centrifuged at 1000*g* for 5 min. Extractions were performed in triplicate and supernatants were pooled in separate tubes, with final volumes for each aliquot adjusted to a final volume of 25 mL.

GS instrumentation and chromatographic conditions

GS products were analyzed by using the protocol of Abourashed and Khan (2001), which was adapted and modified for our equipment. Briefly, all GS samples were analysed on a Varian ProStar 230 component HPLC system (Ghent, Belgium) equipped with a photodiode array detector, column temperature controller, and autosampler. Samples were eluted by gradient from a Waters Symmetry C-18 column (150 mm × 4.6 mm; 5 μm particle size; serial no. T63451L) (Mississauga, Ont.). The mobile phase consisted of 50 mmol/L sodium phosphate (NaH_2PO_4) buffer (pH 2.1) (solvent A) and acetonitrile (solvent B). Solvent A varied from 75% to 25% over the first 15 min and from 25% to 10% at 15–18 min. The solvent flow rate was 1.0 mL/min. The injection volume was 5 μL for all samples tested. The signal was monitored at 295 nm for hydrastine and 350 nm for berberine. Data collection and integration were performed with the accompanying Varian software.

MT instrumentation and chromatographic conditions

The protocol used was adapted and based on the method published online by NSF International (2004). Briefly, all MT samples were analysed on an Agilent 1100 Series HPLC system (Santa Clara, Calif.) equipped with a photodiode array detector, in-line degasser, column temperature controller, and autosampler. Samples were eluted by gradient from a Waters YMC column (50 mm × 2.0 mm; 3 μm particle size; serial No. 1973101521D04) (Mississauga, Ont.). The mobile phase consisted of ddH_2O (solvent A), acetonitrile (solvent B,) and MeOH (solvent C). Solvent C was held constant at 10%, with solvent B varying from 5% to 50% over the first 10 min and from 50% to 90% from 10–12 min. The solvent flow rate was 0.5 mL/min. The injection volume was 1 μL for all the samples tested. Several MT samples were diluted 10-fold before analysis (methanolic and ethanolic extracts of both MT-NRP 126 and NRP18). The signal was monitored at 285 nm for silibinin and the silymarin group (considered to be all peaks from 3.5 min to 11 min). Data collection and integration were performed with the accompanying Hewlett Packard software.

Caco-2 cell culture

Caco-2 cells (C2BBe1: human adenocarcinoma colon cells; clone of Caco-2) were routinely subcultured, grown, and maintained in 50-millilitre cell culture flasks (Nunclon,

Table 2. HPLC analysis for the constituents hydrastine and berberine from selected goldenseal capsules, and for the constituents silibinin and the silymarin group (includes toxifolin, silichristin, silidianin, silybin A, silybin B, isosilybinin A, and isosilybin B) from selected milk thistle capsules.

		Content/capsule, mg	
Herbal product (NRP No.)	Extract solvent	Hydrastine	Berberine
Goldenseal (121)	Water	9.01±0.67	4.31±0.53
	70% Ethanol	13.32±0.75	16.56±0.27
	Methanol	11.85±0.67	15.41±0.67
Goldenseal (17)	Water	29.77±7.91	52.75±1.36
	70% Ethanol	47.30±2.32	59.23±2.76
	Methanol	54.66±0.20	67.18±0.34
		Content/capsule, mg	
Herbal product (NRP No.)	Extract solvent	Silibinin	Silymarin group
Milk thistle (126)	Water	0.50±0.05	3.73±0.30
	70% Ethanol	44.65±5.95	90.42±6.60
	Methanol	78.22±3.98	91.88±11.34
Milk thistle (18)	Water	0.54±0.14	3.13±0.77
	70% Ethanol	45.85±1.50	84.94±1.50
	Methanol	58.25±5.31	74.00±5.49

Note: Berberine content was determined at wavelength 350 nm, hydrastine at 295 nm, and both silibinin and the silymarin group at 285 nm. NRP No., natural research product designation number. Extracts of herbal capsules were prepared in triplicate; values are means ± SD.

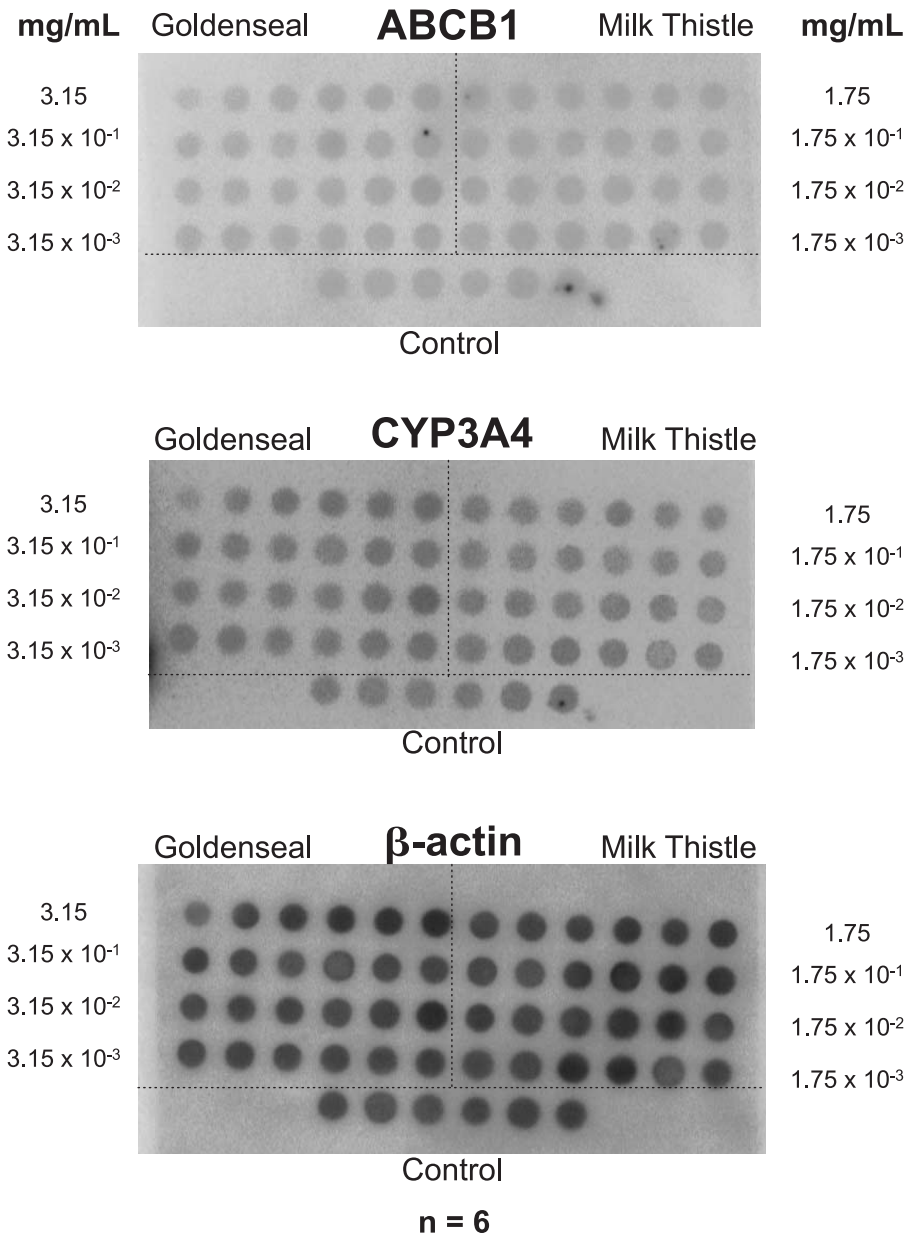
Rochester, N.Y.) in a Fisher Scientific 605 incubator (Ottawa, Ont.) set to 37 °C and 5% CO_2. Culture feeding media consisted of DMEM (Dulbecco's modified Eagle media) supplemented with 20% FBS (fetal bovine serum), 50 units of penicillin–streptomycin per mL of media, and 0.25% human transferrin (lyophilized; 4 mg/mL). All cell culture supplies were obtained from Gibco (Burlington, Ont.). Cell culture health was routinely assessed by the following: (*i*) direct observation of cultured cells via microscopy for contamination; (*ii*) cell density count comparisons; (*iii*) pH measurements; and (*iv*) determination of glucose utilization via analysis with the Glucose Liqui-Color enzymatic-colorimetric test kit (Stanbio, Boerne, Tes.).

For the individual experiments, Caco-2 cells were subcultured and plated at an initial density of 1×10^6 cells in 8 mL of 20% FBS DMEM and allowed to grow for 120 h at 37 °C. Subsequently, the formed Caco-2 cell monolayers were treated for 48 h with media containing either a 10% herbal extract (10× stock solution and corresponding dilutions of 1×, 0.1×, and 0.01×), 10% ddH_2O with a pure compound dilution, or 10% ddH_2O (control). Cells were then washed, trypsinized, and harvested. Briefly, the media from each flask was discarded and the cell monolayer surface rinsed with 5 mL of trypsin EDTA. Cells were trypsinized for 10 min with another 5 mL of trypsin EDTA and then collected in a 15-millilitre centrifuge tube. Ten millilitres of fresh media were added to the tube and the cells were resuspended. A small aliquot (approximately 0.2 mL) was obtained from each cell suspension for the purposes of counting, and the remaining cells were centrifuged at 260*g* for 10 min. The supernatant was then discarded and the remaining cell pellet was flash-frozen in liquid N_2 and stored at –80 °C until needed.

RNA extraction

Total RNA was isolated from frozen pellets of treated or untreated Caco-2 cells by means of the acid guanidinium thiocyanate (GITC)–phenol–chloroform method (Chomczynski and Sacchi 1987; Chomczynski and Mackey 1995). Frozen cell pellets were homogenized for 1 min in 1.0 mL of denaturing solution (solution D, per 100 mL: 73 mL of diethyl pyrocarbonate (DEPC)-treated water, 62.5 g of solid GITC, 4.4 mL of 0.75 mol/L sodium citrate (pH 7.0), 6.6 mL of 10% sarkosyl (10 g of sodium lauryl sarcosinate per 100 mL DEPC-treated water), and 0.3 mL of β-mercaptoethanol) (Sigma Aldrich, St. Louis, Mo.). After homogenization, 0.5 mL of phenol (BDH) saturated with DEPC-treated water was added to the suspension and the solution was vortexed. Subsequently, 0.2 mL of chloroform was added and the resultant solution was vortexed again. The solution was transferred to an RNase-free 2-millilitre microcentrifuge tube and centrifuged at 12 000*g* for 20 min in an Eppendorf (Mississauga, Ont.) microfuge. The supernatant (aqueous phase) was then transferred to a new 2-millilitre microcentrifuge tube and the remaining solution was frozen in liquid N_2 and stored at –80 °C for future protein purification from the organic phase. To the supernatant, 0.6 mL of isopropanol was added and the resultant mixture was vortexed and centrifuged again at 14 000*g* for 15 min. This new supernatant was discarded and the RNA pellet at the bottom of the tube was washed with 1.0 mL of ice-cold 80% EtOH and centrifuged again for 10 min at 14 000*g*. This washing procedure was repeated twice and after the second wash, the RNA pellet was air dried for 10 min and then suspended in 100 µL of DEPC-treated water. The RNA concentration of individual samples was determined by drawing a 2-microlitre aliquot from each and suspending it in 98 µL of filtered 0.1× TE (Tris–EDTA); it was quantified with an Amersham Pharmacia GeneQuant DNA/RNA calculator spectrophotometer

Fig. 1. A representative dot blot membrane probed for *ABCB1* mRNA, *CYP3A4* mRNA, and β-actin mRNA. The membrane shown was blotted with 7 μg of total RNA isolated from Caco-2 cell monolayers treated with a 10-fold dilution series prepared from aqueous extracts of commercial goldenseal (NRP 17) or milk thistle (NRP 18) capsules. Images represent 48 h of exposure of hybridized membranes to a Kodak K-screen imaged with a Bio-Rad phosphorimager.

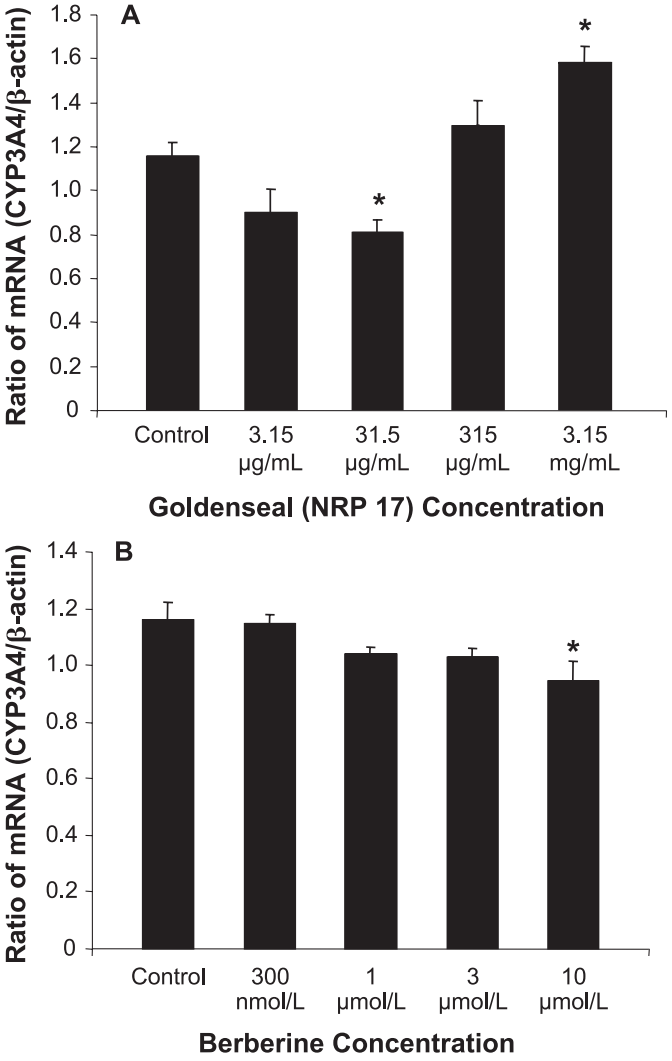


(Little Chalfront, UK). RNA purity was determined from the ratio of absorbances at 260 nm and 280 nm (A_{260}/A_{280}). The remaining stock solution of RNA was frozen in liquid N_2 and stored at –80 °C.

RT-PCR and cDNA synthesis

First-strand cDNA was synthesized from 1–5 μg of total RNA (isolated from untreated Caco-2 cells) by using oligo(dT) primer and Superscript II reverse transcriptase. Specific oligo(dT) primers for *ABCB1* and *β-actin* were designed to amplify and isolate specific fragments of each gene via PCR. Oligoprimers for *ABCB1* were modeled after those used by Pu et al. (1996) and were designed to amplify *ABCB1* cDNA with a product size of 249 nucleotides from exon 13 to exon 15 of the *ABCB1* gene. The sense strand primer sequence was 5′-GGA AGC CAA TGC CTA TGA CT-3′ and the antisense stand primer was 5′-CGA TGA GCT ATC ACA ATG GT-3′. The *β-actin* gene was used to control for variations in RNA loading within the experimental conditions; oligoprimers for *β-actin* were also modeled after those used by Pu et al. (1996). The sense strand primer sequence was 5′-CAT CCT CAC CCT GAA GTA CC-3′ and the antisense stand primer was 5′-GGT GAG GAT CTT CAT GAG GT-3′ with a PCR product size of 394 nucleotides. Individual PCR reactions were carried out in 0.5-millilitre microcentrifuge tubes in a total reaction volume of 50 μL (per reaction: 36.25 μL of DEPC-treated water, 5 μL of 10× PCR buffer, 1.5 μL of 50 mmol/L

Fig. 2. *CYP3A4* mRNA levels for Caco-2 cells treated with varying concentrations of aqueous extracts of (A) goldenseal NRP 17 capsules and (B) berberine. Data are the mean ratios of *CYP3A4* expression relative to β-actin ± SE (n = 18). Means were analysed via a one-way ANOVA followed by Dunnett's 2-sided pairwise comparison, and only data sets with values significantly different ($p < 0.05$) from the control are shown. Aqueous extracts of goldenseal NRP 121 capsules did not affect *CYP3A4* mRNA levels at tested concentrations (6.75 µg/mL, 67.5 µg/mL, 675 µg/mL, 6.75 mg/mL). No significant changes in *ABCB1* mRNA levels were noted compared with control values for both goldenseal products or berberine at tested concentrations.



$MgCl_2$, 1 µL of 10 mmol/L dNTP mix, 2 µL of each oligo(dT) primer (maintained as a 25 µmol/L stock solution), 2 µL of cDNA template, and 0.25 µL of *Taq* polymerase). PCR reaction was performed for either 25 cycles (*ABCB1*) or 20 cycles (*β-actin*) under the following schema: initial denaturation at 95 °C for 5 min, then denaturation at 94 °C for 40 s, annealing at 54 °C (*ABCB1*) or 57 °C (*β-actin*) for 40 s, and extension at 72 °C for 1 min. Amplified products were then size-fractionated by electrophoresis in 50 mL of 1.2% agarose gel and visualized. Bands corresponding to the expected size were excised and purified with the QIAquick gel extraction kit (Qiagen, Mississauga, Ont.). After PCR reactions, purified cDNA was ligated into the pCRII-TOPO cloning vector (Invitrogen, Burlington, Ont.) and used to transform One Shot competent cells (Invitrogen, Burlington, Ont.). Plasmids were subsequently isolated and purified from transformed bacterial cultures with the Wizard Miniprep kit (Promega) and sent to the Canadian Molecular Research Services (Ottawa, Ont.) for sequencing. Nucleotide sequences were subsequently verified via the internet by using GenBank.

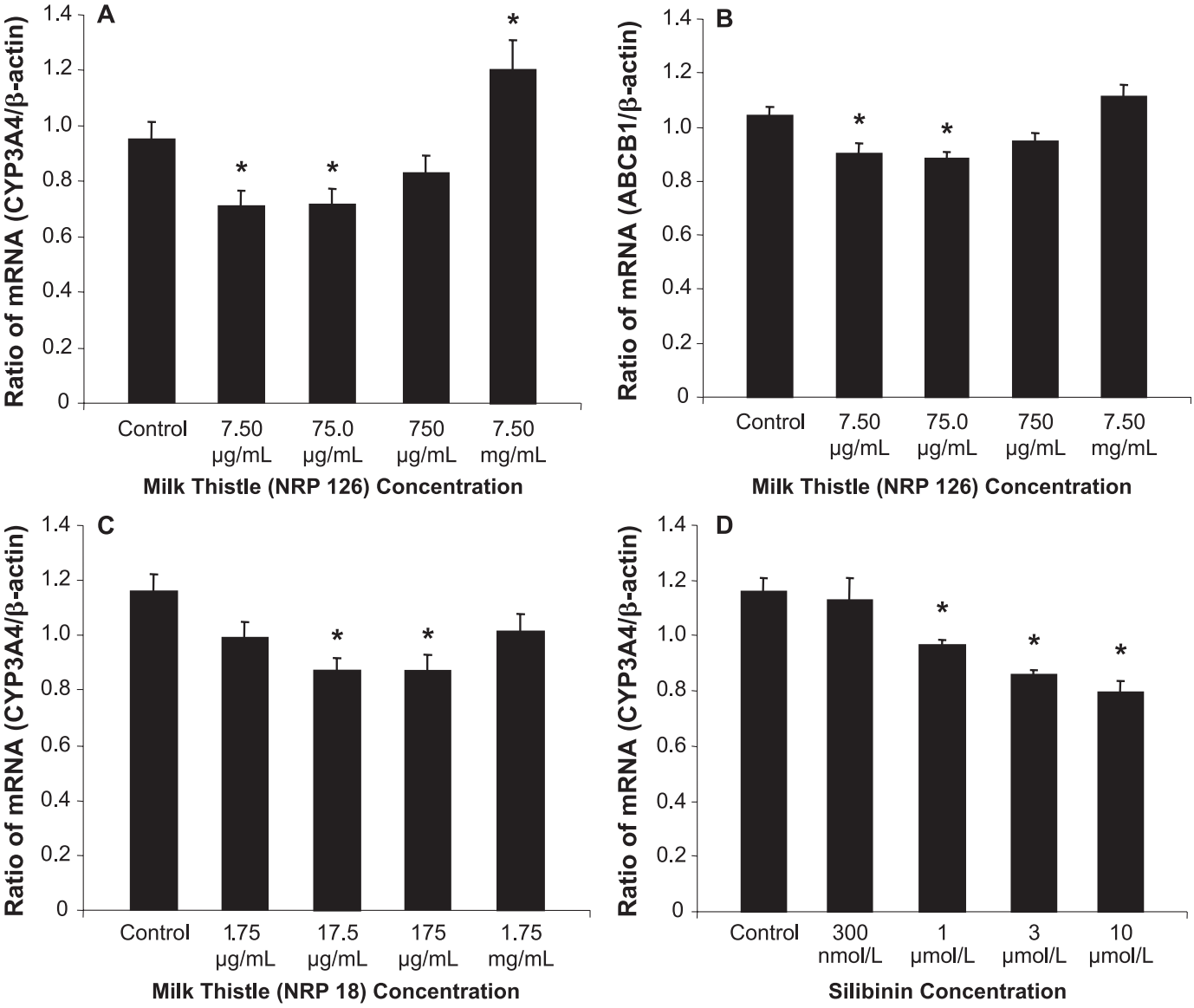
First strand cDNA for *CYP3A4* was also synthesized by using the RT-PCR technique as described earlier. Instead of total RNA isolated from untreated Caco-2 cells serving as a template, however, a *CYP3A4* expression vector (*p*UV1) obtained from Dr. Frank Gonzalez, US National Institutes of Health, was used. The sense strand primer sequence was 5′-CAA GAC CCC TTT GTG GAA AA-3′ and the antisense stand primer was 5′-TCT GAG CGT TTC ATT CAC CA-3′ with a PCR product size of 498 nucleotides. The PCR reaction was performed as previously described for 30 cycles under the following schema: predenaturation at 95 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s, and extension at 72 °C for 1 min. Amplified products were isolated, purified, ligated into pCRII-TOPO, used to transform competent cells, and ultimately sent for sequencing as previously described.

Dot blotting and hybridization

Total RNA (7 µg) isolated from various Caco-2 cell treatments was denatured in 8 µL of denaturation buffer per microgram of RNA at 65 °C for 10 min. RNA denaturation buffer consisted of 50% deionized formamide (Sigma Aldrich, St. Louis, Mo.), 2.2 mol/L formaldehyde (BDH), 20 mmol/L MOPS (morpholinepropanesulfonic acid) (pH 7.0), 5 mmol/L sodium acetate, and 1 mmol/L disodium EDTA. RNA was chilled on ice and diluted 1:2 (*v*/*v*) with 20× SSC (standard sodium citrate). The final volume was then adjusted to 0.5 mL with 10× SSC before loading. RNA was loaded onto Hybond N+ nylon membranes (Amersham Pharmacia Biotech, Little Chalfront, UK) presoaked with 10× SSC by using a Schleicher and Schuell Minifold I dot blot apparatus. The RNA was applied to each well from the resultant 0.5 mL of solution and then subsequently rinsed twice with 0.5 mL of 10× SSC. Membranes were allowed to dry under vacuum for approximately 10 min and then subjected to intense UV light for 5 min to covalently bind the RNA to the membrane.

For hybridization, membranes were washed twice in a solution of 0.1× SSC and 0.1% SDS (sodium dodecyl sulfate) for 20 min each at 65 °C. Hybridization solution (10% dextran sulfate, 1% SDS, 6× SSC, 100 µg/mL of salmon sperm DNA, 5× Denhardt's solution) was added (10 mL per 100 cm^2 of membrane) and the membrane was prehybridized for 3 h at 65 °C. During the prehybridization, radiolabelled probes were prepared by the following method: (*i*) diluting 25 ng of the appropriate template cDNA in 45 µL of 1X TE (10 mmol/L Tris, 1.0 mmol/L EDTA); (*ii*) denaturing the cDNA by placing the mixture at 100 °C for 5 min; (*iii*) radiolabelling the cDNA by means of Redivue stabilized [α^{32}P]dCTP and the Rediprime II random prime DNA labelling system (Amersham Pharmacia Biotech, Little Chalfront, UK); and (*iv*) purifying the radiolabelled probe by

Fig. 3. (A) *CYP3A4* mRNA and (B) *ABCB1* mRNA levels for Caco-2 cells treated with varying concentrations of aqueous extracts of milk thistle NRP 126 capsules. *CYP3A4* mRNA levels for similar treatments with (C) aqueous extracts of milk thistle NRP 18 capsules and (D) silibinin. Data are the mean ratios of *ABCB1* or *CYP3A4* expression relative to β-actin ± SE (n = 18). Means were analysed via a one-way ANOVA followed by Dunnett's 2-sided pairwise comparison, and only data sets with values significantly different ($p < 0.05$) from the control are shown. Aqueous extracts of milk thistle NRP 18 and silibinin did not affect *ABCB1* mRNA levels compared with control values at tested concentrations.



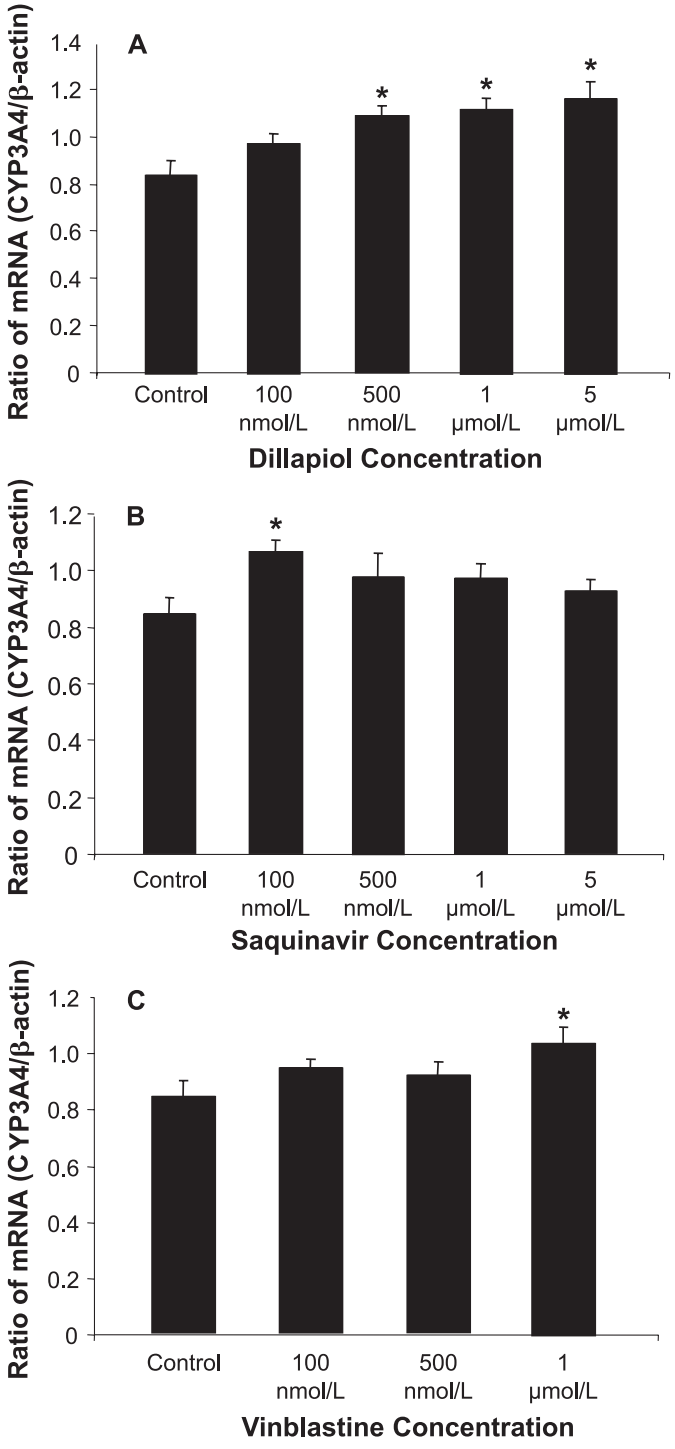
using a Probe Quant G-50 Micro Column (Amersham Pharmacia Biotech, Little Chalfront, UK) to remove unbound radioactive nucleotides. After the prehybridization was complete, the hybridization solution was replaced with an equal volume of fresh solution and supplemented with the radiolabelled probe. The mixture was left to hybridize overnight for approximately 18 h at 65 °C. Membranes were subsequently washed at 65 °C as follows: (*i*) rinsed vigorously with 1× SSC and 0.1% SDS; (*ii*) 10 min with 1× SSC and 0.1% SDS; and (*iii*) 10 min with 0.1× SSC and 0.1% SDS. Membranes were then placed on Whatman filter paper, wrapped in plastic food wrap, and exposed to a Kodak K phosphor screen for 48 h. Phosphor screens were scanned with a Bio-Rad (Hercules, Calif) phosphor imager and dot intensities analyzed with the Bio-Rad Quantity One quantitation software (release 4).

Hybridizations for all membranes occurred in the following order: ABCB1, CYP3A4, and β-actin. Between hybridizations, membranes were stripped of all radioactivity by washing them with 1% SDS for 20 min at 85 °C. A lack of radioactivity was verified by observing membranes after a short-term exposure to Kodak K screens (6 h) and visualizing them as previously described.

Statistical analysis

For a given membrane, the densitometric data for the relative expression of *ABCB1*and *CYP3A4* (corrected for β-actin

Fig. 4. *CYP3A4* mRNA levels for Caco-2 cells treated with varying concentrations of pure compounds (A) dillapiol, (B) saquinavir, and (C) vinblastine. Data are the mean ratios of *CYP3A4* expression relative to β-actin ± SE (n = 18). Means were analysed via a one-way ANOVA followed by Dunnett's 2-sided pairwise comparison, and only data sets with values significantly different ($p < 0.05$) from the control are shown. No significant changes in *ABCB1* mRNA levels were noted compared with control values for both goldenseal products or berberine at tested concentrations.



to control for variation in loading) was calculated as the (counts × mm^2 for each blot)/(mean counts × mm^2 for all blots). This accounted for differences among membranes, and thus ratios from multiple membranes were then pooled and analyzed via a one-way ANOVA, followed by Dunnett's two-sided pairwise comparison (significance set to $p < 0.05$) carried out with Systat statistical software (version 11, SPSS Inc., Chicago, Ill.).

Protein isolation and Western blot analysis

The total protein was recovered from the organic phase leftover during the RNA isolation previously described. Briefly, 0.8 volume of 100% EtOH was added to the organic phase and after centrifugation at 2000*g* for 5 min at 4 °C, 2 volumes of isopropanol were added to the supernatant. After centrifugation at 13 000*g* for 10 min at 4 °C, the pellet was washed by 90% EtOH 3 times. The pellet was subsequently air dried and dissolved in 1% SDS. The protein concentration was then determined by means of Bio-Rad's protein assay (Hercules, Calif.) with bovine serum albumin (BSA) serving as a standard.

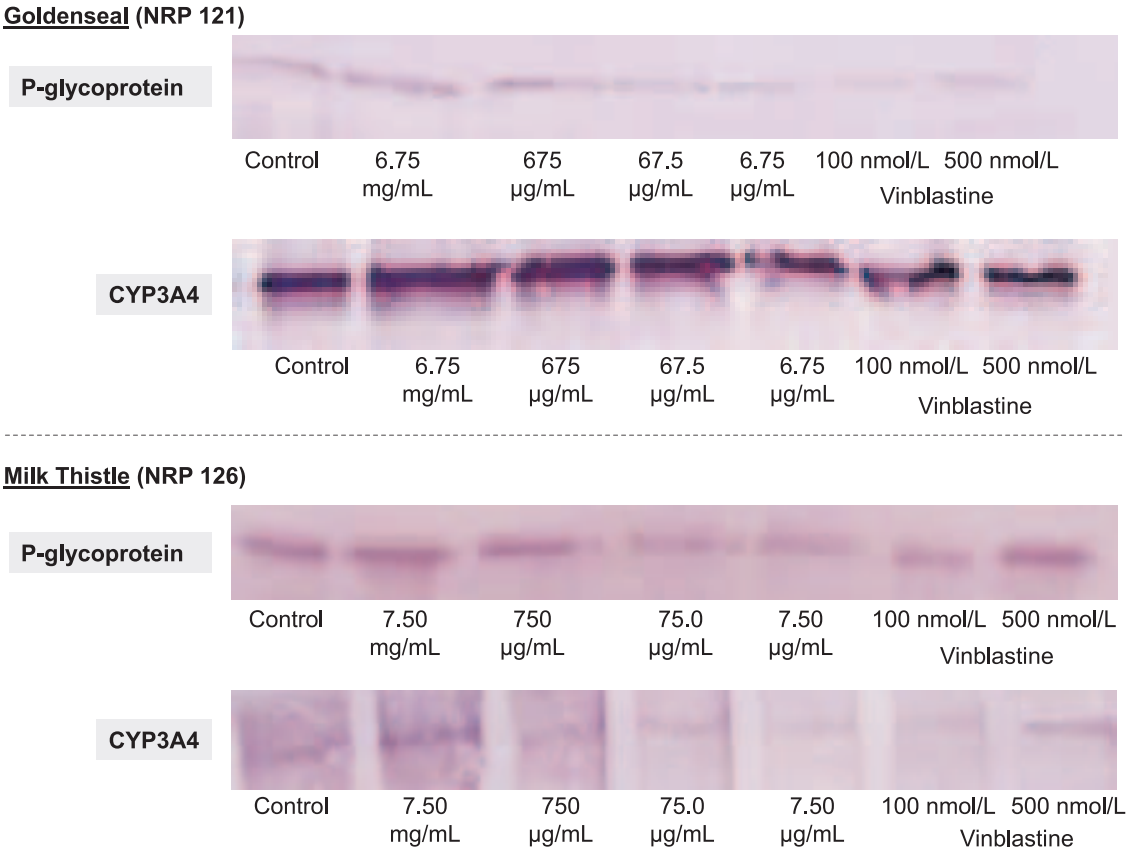
Samples were subjected to electrophoresis on a 7.5% polyacrylamide gel and transferred to nitrocellulose with standard techniques. The blot was blocked in 3% nonfat milk and incubated with primary antibody: C494 (ID Labs Inc., London, Ont., 1:500 dilution) for *ABCB1* and CYP3A4 Mabr (Research Diagnostics Inc., Concord, Mass., 1:500 dilution) for *CYP3A4*. The blot was then washed with TTBS (Tris-buffered saline containing 0.05% polysorbate 20) and incubated with secondary alkaline phosphatase-conjugated antibody: goat anti-mouse for *ABCB1* (Sigma Aldrich, St. Louis, Mo.) or goat anti-rabbit for *CYP3A4* (Bio-Rad, Hercules, Calif.) with a dilution of 1:3000. The signal was observed by using tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate and was quantified with a Kodak image scan program. Each Western blot was repeated 2 times per sample set, and owing to the low amount of protein recovered, organic fractions were pooled from multiple experiments (N = 1).

Results

HPLC analysis of GS and MT NHPs

GS and MT capsules were standardized for their respective marker compounds via an HPLC analysis of 3 prepared solvent preparations from each preparation (i.e., water (deionized/distilled), 70% ethanol, and methanol). GS-prepared extracts were analyzed for levels of the alkaloids hydrastine and berberine; MT-prepared extracts were analyzed for levels of the flavonolignan constituents silibinin and the silymarin group (i.e., total silymarins). Overall, it was noted that aqueous extracts for all products yielded considerably lower levels of their respective marker compounds than those of alcoholic extracts. Among the tested GS products, only GS-NRP 17 had specific label claims for total alkaloid content (10%), and this potency was supported by the HPLC analysis of the 3 tested prepared GS-NRP 17 extracts. Total silymarins were found to be approximately 19% for the tested prepared MT extracts, despite label claims of 80%. These results are highlighted in Table 2.

Fig. 5. Representative Western blot membranes showing protein levels of P-glycoprotein (P-gp) and *CYP3A4*. Bands correspond to the pooled total protein ($N = 1$) recovered from the organic phase leftover during the total RNA isolation protocol. Bands correspond to Caco-2 cell treatments with varying concentrations of aqueous extracts of goldenseal NRP 121 capsules or milk thistle NRP 126 capsules.



Expression of *ABCB1* and *CYP3A4*

Membranes dotted with total RNA isolated from Caco-2 cells treated with NHP extracts or pure compounds were hybridized with a radiolabelled cDNA probe for the specific mRNAs. Figure 1 highlights some typical representative dot blot membranes. It was consistently observed that the levels of relative gene expression among all of the membranes were as follows: *ABCB1* < *CYP3A4* < β-actin. Densitometric data were compared among multiple membranes, grouped, and summarized by experimental treatment (Figs. 2, 3, and 4).

After 48 h of exposure, extracts of GS-NRP 17 had no significant effect on *ABCB1* or *CYP3A4* expression for any of the tested concentrations. Extracts of GS-NRP 121 did not significantly affect *ABCB1* expression, and there was an observed bimodal modulation for these extracts on levels of *CYP3A4* mRNA (Fig. 2); full strength GS-NRP 121 (6.75 mg/mL) extracts upregulated *CYP3A4* expression, whereas GS-NRP 121 (67.5 µg/mL) extracts downregulated *CYP3A4* expression. MT-NRP 18 extracts did not have any significant effect on *ABCB1* expression, but 2 extract dilutions (175 µg/mL and 17.5 µg/mL) significantly downregulated expression of *CYP3A4* (Fig. 3). It is interesting to note that levels of *ABCB1* mRNA in the Caco-2 cells were not significantly altered by GS-NRP 121 treatments; a similar but nonsignificant trend of bimodal modulation was observed as with *CYP3A4* expression. A similar bimodal effect on *CYP3A4* expression was observed with MT-NRP 126 extract treatments (Fig. 3), with an upregulation occurring for MT-NRP 126 (7.75 mg/mL) and a downregulation occurring for MT-NRP 126 (77.5 µg/mL and 7.75 µg/mL). MT-NRP 126 extracts also decreased levels of *ABCB1* mRNA for the 2 lowest tested concentrations (77.5 µg/mL and 7.75 µg/mL).

Pure compounds including phytochemicals and the anti-HIV drug saquinavir were also examined for their ability to alter gene expression in treated Caco-2 cell monolayers. Various concentrations of the GS alkaloid berberine (Fig. 2) had no significant effect on *ABCB1* expression, although *CYP3A4* was downregulated by the highest exposure concentration (10 µmol/L). The MT flavonolignan silibinin (Fig. 3) also had no appreciable effect on *ABCB1* expression, but a clear dose-dependent downregulation of *CYP3A4* was observed. The vinca alkaloid and known P-gp inhibitor vinblastine (Parasrampuria et al. 2001) did not affect levels of *ABCB1* mRNA, but did upregulate *CYP3A4* expression for treatments at 1 µmol/L (Fig. 4). The HIV protease inhibitor saquinavir had no effect on *ABCB1* nor *CYP3A4* expression, except an apparent increase of *CYP3A4* mRNA at the lowest tested concentration (100 nmol/L) (Fig. 4). Dillapiol, a compound occurring in dill and *Piper* sp. and a known *CYP3A4* inhibitor (Budzinski et al. 2000), was found to significantly upregulate *CYP3A4* expression in a dose-dependent manner, and had no appreciable effect on *ABCB1* mRNA levels at the concentrations tested (Fig. 4).

Table 3. Relative protein levels of P-glycoprotein and CYP3A4 isolated from Caco-2 cells treated with goldenseal and milk thistle capsule extracts or pure compounds.

		Relative protein level vs. control	
Herbal product (NRP No.)	Treatment, mg/mL	P-gp	CYP3A4
Goldenseal (17)	3.15	0.18	0.32
	3.15×10^{-1}	0.28	0.43
	3.15×10^{-2}	0.37	0.50
	3.15×10^{-3}	2.62	0.46
Goldenseal (121)	6.75	1.65	1.26
	6.75×10^{-1}	1.38	1.16
	6.75×10^{-2}	0.79	0.88
	6.75×10^{-3}	0.59	0.72
Milk thistle (18)	1.75	2.00	0.38
	1.75×10^{-1}	1.57	0.10
	1.75×10^{-2}	1.92	0.56
	1.75×10^{-3}	3.00	1.08
Milk thistle (126)	7.75	1.16	1.70
	7.75×10^{-1}	0.80	0.84
	7.75×10^{-2}	0.58	0.70
	7.75×10^{-3}	0.50	0.49
		Relative protein level vs. control	
Pure compound	Treatment, μmol/L	P-gp	CYP3A4
Berberine	10	1.02×10^{1}	1.64
	3	1.26	1.14
	1	4.32	0.42
	0.3	1.12	0.47
Silibinin	10	1.69	0.91
	3	1.04×10^{1}	1.60
	1	1.99	2.25
	0.3	4.58	1.01
Dillapiol	5	1.52	0.91
	1	0.31	0.81
	0.5	2.85	0.86
	0.1	0.76	0.45
Saquinavir	5	2.99	3.49
	1	0.79	2.65
	0.5	0.53	2.21
	0.1	0.32	1.58
Vinblastine	1	0.42	0.30
	0.5	0.38	0.21
	0.1	0.29	0.19

Note: P-gp, P-glycoprotein. Protein levels from each experiment were pooled samples ($n = 6$) and are compared with control values.

Protein levels of P-gp and CYP3A4

Protein fractions for all of the experiments were collected from the organic phase of the RNA isolation protocol, purified, pooled, and subjected to immunoblot analysis (Fig. 5). Protein levels from each treatment were expressed as a ratio to the corresponding control value and summarized in Table 3. Relative to the control, levels of CYP3A4 ranged from 0.10 (MT-NRP 18, 1.75×10^{-1} mg/mL) to 2.25 (silibinin, 1 μmol/L). Similarly, levels of P-gp were apparently affected to a larger degree ranging from 0.18 (GS-NRP 17, 3.15 mg/mL) to 10.4 (silibinin, 3 μmol/L). The overall change in levels of mRNA for *ABCB1* and *CYP3A4* are compared with the observed changes in protein in a qualitative manner summarized in Table 4.

Discussion

The interaction of NHP constituents with *CYP3A4* and P-gp can potentially alter the activity of drug-metabolizing enzymes and transporters, thereby leading to changes in the pharmacokinetics of coadministered drugs and other NHPs (Foster et al. 2005). Numerous in vitro and in vivo methodologies exist for assessing which NHP–drug interactions may be of potential clinical significance, but in vivo studies remain the ultimate way for determining the clinical importance of these interactions (Venkataramanan et al. 2006). These studies, however, are expensive, time consuming, and labour intensive. The goal of in vitro studies such as the one described herein is a rapid, relatively inexpensive, high-throughput risk assessment for determining the NHPs (or their constituents) more likely to lead to adverse events, if taken concomitantly with drugs, as a result of competing interactions on common drug disposition mechanisms. Furthermore, the results of in vitro studies may be useful for selecting specific NHPs or NHP preparations for more advanced in vitro experiments and (or) in vivo pharmacokinetic clinical studies. In fact, the GS and MT products used in this study were selected on the basis of results of a concurrent study investigating P-gp and ATPase activity (Budzinski 2003); GS and MT teas also had significantly higher P-gp and ATPase activities than 5 other herbal teas and the positive control verapamil (Budzinski et al. 2001).

It is noted that berberine and silibinin appear to be present in biologically relevant concentrations in the extracts used in the current study. Aqueous extracts of GS and MT products were analyzed for their constituents by HPLC (Budzinski 2003). Caco-2 cells treated in the current study with aqueous GS extract (concentrations ranging from 6.75 mg/mL to 6.75 μg/mL for NRP 121, and 3.15 mg/mL to 3.15 μg/mL for NRP 17) had estimated berberine concentrations in the media ranging from 65.5 μg/mL to 65.5 ng/mL for NRP 121 and from 487.3 μg/mL to 487.3 ng/mL for NRP 17. Both sets of GS extract dilutions encompassed biologically relevant berberine concentrations, as evidenced by plasma concentrations determined by Zeng and Zeng (1999) who observed that berberine plasma concentrations in patients varied from 0.07 μg/mL to 27.1 μg/mL across a variety of conditions. Similarly, Caco-2 cells treated with aqueous MT had extract concentrations ranging from 7.50 mg/mL to 7.50 μg/mL for NRP 126, and 1.75 mg/mL to 1.75 μg/mL for NRP 18. The corresponding estimated silibinin concentrations in the media thus ranged from 7.50 μg/mL to 7.50 ng/mL for NRP 126, and from 2.1 μg/mL to 2.1 ng/mL for NRP 18. In their review of MT usage as a therapy for the treatment of liver disease, Flora et al. (1998) report some maximal silibinin plasma concentrations ranging from 0.12 μg/mL to 0.34 μg/mL; therefore, the extract dilutions utilized in cell treatments encompassed biologically relevant silibinin concentrations.

Table 4. Qualitative summary of mRNA expression for *ABCB1* and *CYP3A4*, as well as their corresponding protein (P-glycoprotein and CYP3A4) levels, compared with basal levels (control) for all experiments.

		mRNA levels		Protein levels	
Herbal product (NRP No.)	Treatment, mg/mL	*ABCB1*	*CYP3A4*	P-gp	CYP3A4
Goldenseal (17)	3.15	NC	NC	Decreased	Decreased
	3.15×10^{-1}	NC	NC	Decreased	Decreased
	3.15×10^{-2}	NC	NC	Decreased	Decreased
	3.15×10^{-3}	NC	NC	Increased	Decreased
Goldenseal (121)	6.75	NC	Increased	Increased	NC
	6.75×10^{-1}	NC	NC	Increased	NC
	6.75×10^{-2}	NC	Decreased	Decreased	NC
	6.75×10^{-3}	NC	NC	Decreased	Decreased
Milk thistle (18)	1.75	NC	NC	Increased	Decreased
	1.75×10^{-1}	NC	Decreased	Increased	Decreased
	1.75×10^{-2}	NC	Decreased	Increased	Decreased
	1.75×10^{-3}	NC	NC	Increased	NC
Milk thistle (126)	7.75	NC	Increased	NC	Increased
	7.75×10^{-1}	NC	NC	NC	NC
	7.75×10^{-2}	Decreased	Decreased	Decreased	Decreased
	7.75×10^{-3}	Decreased	Decreased	Decreased	Decreased
		mRNA levels		Protein levels	
Pure compound	Treatment, μmol/L	*ABCB1*	*CYP3A4*	P-gp	CYP3A4
Berberine	10	NC	Decreased	Increased	Increased
	3	NC	NC	NC	NC
	1	NC	NC	Increased	Decreased
	0.3	NC	NC	NC	Decreased
Silibinin	10	NC	Decreased	Increased	NC
	3	NC	Decreased	Increased	Increased
	1	NC	Decreased	Increased	Increased
	0.3	NC	NC	Increased	NC
Dillapiol	5	NC	Increased	Increased	NC
	1	NC	Increased	Decreased	NC
	0.5	NC	Increased	Increased	NC
	0.1	NC	NC	Decreased	Decreased
Saquinavir	5	NC	NC	Increased	Increased
	1	NC	NC	Decreased	Increased
	0.5	NC	NC	Decreased	Increased
	0.1	NC	Increased	Decreased	Increased
Vinblastine	1	NC	Increased	Decreased	Decreased
	0.5	NC	NC	Decreased	Decreased
	0.1	NC	NC	Decreased	Decreased

Note: P-gp, P-glycoprotein. Because protein levels were derived from pooled samples ($n = 6$), no change (NC) was assigned to treatments falling within 25% of control levels.

In this in vitro cell-based study, Caco-2 cell monolayers were treated with a range of concentrations of aqueous GS and MT extracts, along with berberine, silibinin, dillapiol, vinblastine, and saquinavir. Levels of *ABCB1* and *CYP3A4* expression were measured in response to the treatments. The Caco-2 cell line is considered an industry standard for modeling human drug disposition processes and the intestinal absorption of new compounds (Cummins et al. 2001). In general, rates of drug permeation across Caco-2 cell monolayers correlates well with the amount of passive drug absorption and actively transported compounds, since Caco-2 cells endogenously express numerous types of efflux transporters (P-gp being the predominant one). According to Cummins et al. (2001), there are 2 Caco-2 cell models that express *CYP3A4* at high enough levels to allow for the concurrent study of transport and metabolism. These models are (*i*) Caco-2 cells in which levels of *CYP3A4* have been artificially upregulated or "enhanced" via the addition of 1α,25-dihydroxy vitamin-D_3 to the medium and Caco-2 cells transfected with the *CYP3A4* gene (such as those produced by Gentest). The Caco-2 cell model used in this study was neither of these 2 models, since the goal of the experiment was to determine whether or not *CYP3A4* and *ABCB1* could be modulated from an endogenous state. Transport studies

were not performed because the total RNA was isolated from stripped and trypsinized monolayers. *CYP3A4* was found to be highly responsive in our system as evidenced by a strong dose-dependent modulation (e.g., an observed upregulation by dillapiol and downregulation by silibinin). Perhaps this is one reason for the poor responsiveness of *ABCB1* apparent in this system. Given that P-gp and *CYP3A4* have numerous overlapping substrate specificities (Cummins et al. 2002), it is conceivable that the majority of P-gp substrates, especially in the MT and GS extracts, were extensively metabolized by *CYP3A4* and thus had less influence on *ABCB1* and its gene product. A longer-term exposure and (or) chronic repeat-dose study in this system would be useful for better characterizing the modulation of *ABCB1* gene expression. Currently, due to the fact that the protein fractions from each treatment were pooled for Western blot analysis ($N = 1$), it is difficult to draw any conclusions comparing the observed changes in *ABCB1* or *CYP3A4* mRNA with the corresponding protein levels (although the trends have been described). More studies need to be completed to reevaluate and confirm these results.

Although many xenobiotics can influence CYP P450 metabolism, and there is considerable information available on *CYP3A4* inhibition by NHPs, there is little information available on the gene induction of the key drug disposition mechanisms *CYP3A4* and *ABCB1* (P-gp) by these 2 commonly used herbs or their constituents. As such, this study is warranted and original, and it represents an initial investigation into the area of potential risk assessment. In general, in vitro studies such as these allow for comparative risk assessment among numerous products, since not all NHPs or specific formulations are likely to have the same inhibitory capability of *CYP3A4* or other P450 enzymes, influence the P-gp transport of overlapping substrates to the same degree, or result in significant changes in gene expression or subsequent changes to levels of the expressed gene product. Our lab has conducted several experiments involving GS and MT products, and based on the current, and previous studies (Budzinski 2003), important markers for the in vitro assessment of NHP–drug interactions are (*i*) the modulation of *CYP3A4* gene expression, (*ii*) the inhibition of *CYP3A4* activity, and (*iii*) the stimulation of P-gp ATPase activity. Selected NHPs showing both high and low activity in any one of these assays need to be subject to more detailed in vivo pharmacokinetic clinical studies to determine whether the current in vitro results correlate to the in vivo state.

Acknowledgements

The author thanks Marwan Samia for his molecular biology expertise and guidance, Dr. Frank Gonzalez at the US National Institutes of Health for providing our group with a *CYP3A4* expression vector (*p*UV1), and Dr. Rolf van Heeswijk at the Ottawa Hospital for providing our group with a stock solution of saquinavir. Funding for this research was provided by the Canadian Association for HIV Research, Positive Action Program, AIDS Bureau, Ontario Ministry of Health and Long-Term Care; the Natural Sciences and Engineering Research Council of Canada (Strategic Program); and Health Canada.

References

Abourashed, E.A., and Khan, I.A. 2001. High-performance liquid chromatography determination of hydrastine and berberine in dietary supplements containing goldenseal. J. Pharm. Sci. Technol. **90**: 817–822. doi:10.1002/jps.1035.

Ameer, B., and Weintraub, R.A. 1997. Drug interactions with grapefruit juice. Clin. Pharmacokinet. **33**: 103–121. PMID: 9260034.

Bernard, C.B., Krishnamurty, H.G., Chauret, D., Sanchez, P.E., San Roman, L., Poveda, L.J., and Hasbrun, C. 1995. Insecticidal Piperaceae of the neotropics. J. Chem. Ecol. **21**: 801–814. doi:10.1007/BF02033462.

Budzinski, J.W. 2003. *In vitro* interactions of natural health products with P-glycoprotein (P-gp) and cytochrome P450 3A4 (CYP3A4): Relevance to HIV Therapies. M.Sc. thesis, Ottawa-Carleton Institute of Biology, University of Ottawa, Ottawa, ON.

Budzinski, J.W., Foster, B.C., Vandenhoek, S., and Arnason, J.T. 2000. An in vitro analysis of human cytochrome P450 3A4 inhibition by selected commercial herbal extracts and tinctures. Phytomedicine, **7**: 273–282. PMID:10969720.

Budzinski, J.W., Arnason, J.T., Trudeau, V.L., Krantis, A., and Foster, B.C. 2001. Effects of goldenseal and milk thistle on the modulation of cytochrome P50 3A4 and P-glycoprotein. Drug Metab. Rev. **33**(S1): 86.

Chatterjee, P., and Franklin, M.R. 2003. Human cytochrome P450 inhibition and metabolic-intermediate complex formation by goldenseal extract and its methylenedioxyphenyl components. Drug Metab. Dispos. **31**: 1391–1397. doi:10.1124/dmd.31.11.1391. PMID:14570772.

Chomczynski, P., and Mackey, K. 1995. Short technical reports. Modification of TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. Biotechniques, **19**: 942–945. PMID:8747660.

Chomczynski, P., and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**: 156–159. doi:10.1016/0003-2697(87)90021-2. PMID:2440339.

Cummins, C.L., Mangravite, L.M., and Benet, L.Z. 2001. Characterizing the expression of *CYP3A4* and efflux transporters (P-gp, MRP1, and MRP2) in CYP3A4-transfected Caco-2 cells after induction with sodium butyrate and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate. Pharm. Res. **18**: 1102–1109. doi:10.1023/A:1010914624111. PMID:11587480.

Cummins, C.L., Jacobsen, W., and Benet, L.Z. 2002. Unmasking the dynamic interplay between intestinal P-glycoprotein and CYP3A4. J. Pharmacol. Exp. Ther. **300**: 1036–1045. doi:10.1124/jpet.300.3.1036. PMID:11861813.

Flora, K., Hahn, M., Rosen, H., and Benner, K. 1998. Milk thistle (*Silybum marianum*) for the therapy of liver disease. Am. J. Gastroenterol. **93**: 139–143. doi:10.1111/j.1572-0241.1998.00139.x. PMID:9468229.

Foster, B.C., Foster, M.S., Vandenhoek, S., Krantis, A., Budzinski, J.W., Arnason, J.T., Gallicano, K.D., and Choudri, S. 2001. An in vitro evaluation of human cytochrome P450 3A4 and P-glycoprotein inhibition by garlic. J. Pharm. Pharm. Sci. **4**: 176–184. PMID:11466175.

Foster, B.C., Arnason, J.T., and Briggs, C.J. 2005. Natural health products and drug disposition. Annu. Rev. Pharmacol. Toxicol. **45**: 203–226. doi:10.1146/annurev.pharmtox.45.120403.095950. PMID:15822175.

Gurley, B.J., Gardner, S.F., Hubbard, M.A., Williams, D.K., Gentry, W.B., Carrier, J., et al. 2004. In vivo assessment of botanical supplementation on human cytochrome P450 phenotypes: *Citrus aurantium, Echinacea purpurea*, milk thistle, and saw

palmetto. Clin. Pharmacol. Ther. **76**: 428–440. doi:10.1016/j.clpt.2004.07.007. PMID:15536458.

Gurley, B.J., Gardner, S.F., Hubbard, M.A., Williams, D.K., Gentry, W.B., Kahn, I.A., and Shah, A. 2005. In vivo effects of goldenseal, kava kava, black cohosh, and valerian on human cytochrome P450 1A2, 2D6, 2E1, and 3A4/5 phenotypes. Clin. Pharmacol. Ther. **77**: 415–426. doi:10.1016/j.clpt.2005.01.009. PMID:15900287.

Harborne, J.B., and Baxter, H. 1993. Phytochemical dictionary: a handbook of bioactive compounds from plants. Taylor & Francis, London.

Kivistö, K.T., Niemi, M., and Fromm, M.F. 2004. Functional interaction of intestinal *CYP3A4* and p-glycoprotein. Fundam. Clin. Pharmacol. **18**: 621–626. doi:10.1111/j.1472-8206.2004.00291.x. PMID:15548232.

Lee, L.S., Andrade, A.S.A., and Flexner, C. 2006. Ineractions between natural health products and antiretroviral drugs: pharacokinetic and pharmacodynamic effects. Clin. Infect. Dis. **43**: 1052–1059. doi:10.1086/507894. PMID:16983620.

Maitrejean, M., Comte, G., Barron, D., El Kirat, K., Conseil, G., and Di Pietro, A. 2000. The flavanolignan silybin and its hemisynthetic derivatives, a novel series of potential modulators of P-glycoprotein. Bioorg. Med. Chem. Lett. **10**: 157–160. doi:10.1016/S0960-894X(99)00636-8. PMID:10673101.

Mills, E., Montori, V., Perri, D., Phillips, E., and Koren, G. 2005. Natural health product-HIV drug interactions: a systematic review. Int. J. STD AIDS, **16**: 181–186. doi:10.1258/0956462053420103. PMID:15829016.

Mouly, S.J., Paine, M.F., and Watkins, P.B. 2004. Contributions of CYP3A4, P-glycoprotein, and serum protein binding to the intestinal first-pass extraction of saquinavir. J. Pharmacol. Exp. Ther. **308**: 941–948. doi:10.1124/jpet.103.056390. PMID:14718607.

NSF International. 2004. (Institute for Nutraceutical Advancement). Determination of flavonol derivatives (silymarin group) in milk thistle fruit and powdered extract (silymarins in milk thistle by HPLC - INA method 115.000) [online]. Available from http://www.nsf.org/business/ina/milkthistle.asp?program=INA. [accessed 1 June 2007].

Parasrampuria, D.A., Lantz, M.V., and Benet, L.Z. 2001. A human lymphocyte based *ex vivo* assay to study the effect of drugs on P-glycoprotein (P-gp) function. Pharm. Res. **18**: 39–44. doi:10.1023/A:1011070509191. PMID:11336351.

Patel, J., Buddha, B., Dey, S., Pal, D., and Mitra, A.K. 2004. In vitro interaction of the HIV protease inhibitor ritonavir with herbal constituents: changes in p-gp and *CYP3A4* activity. Am. J. Ther. **11**: 262–277. doi:10.1097/01.mjt.0000101827.94820.22. PMID:15266218.

Piscitelli, S.C., Burstein, A.H., Welden, N., Gallicano, K., and Falloon, J. 2002*a*. The effect of garlic supplements on the pharmacokinetics of saquinavir. Clin. Infect. Dis. **34**: 234–238. doi:10.1086/324351. PMID:11740713.

Piscitelli, S.C., Formentini, R.N.C., Burstein, A.H., Alfaro, R., Jagannatha, S., and Falloon, J. 2002*b*. Effect of milk thistle on the pharmacokinetics of indinavir in healthy volunteers. Pharmacotherapy, **22**: 551–556. doi:10.1592/phco.22.8.551.33205. PMID:12013352.

Pu, Y.S., Tsai, T.C., Cheng, A.L., Tsai, C.Y., Tseng, N.F., Su, I.J., Hsieh, C.Y., and Lai, M.K. 1996. Expression of MDR-1 gene in transitional cell carcinoma and its correlation with chemotherapy response. J. Urol. **156**: 271–275. doi:10.1016/S0022-5347(01)66015-4. PMID:8648821.

Sandhu, R.S., Prescilla, R.P., Simonelli, T.M., and Edwards, D.J. 2003. Influence of goldenseal root on the pharmacokinetics of indinavir. J. Clin. Pharmacol. **43**: 1283–1288. doi:10.1177/0091270003258660.

Sridar, C., Goosen, T.C., Kent, U.M., Williams, J.A., and Hollenburg, P.F. 2004. Silybin inactivates cytochromes P450 3A4 and 2C9 and inhibits major hepatic glucuronosyltrasferases. Drug Metab. Dispos. **32**: 587–594. doi:10.1124/dmd.32.6.587. PMID:15155549.

Stermitz, F.R., Lorenz, P., Tawara, J.N., Zenewicz, L.A., and Lewis, K. 2000. Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5′-methoxyhydnocarpin, a multidrug pump inhibitor. Proc. Natl. Acad. Sci. U.S.A. **97**: 1433–1437. doi:10.1073/pnas.030540597. PMID:10677479.

Thummel, K.E., and Wilkinson, G.R. 1998. *In vitro* and *in vivo* drug interactions involving CYP3A. Annu. Rev. Pharmacol. Toxicol. **38**: 389–430. doi:10.1146/annurev.pharmtox.38.1.389. PMID:9597161.

Venkataramanan, R., Ramachandran, V., Komoroski, B.J., Zhang, S., Schiff, P.L., and Strom, S.C. 2000. Milk thistle, a herbal supplement, decreases the activity of *CYP3A4* and uridine diphosphoglucuronosyltransferase in human hepatocyte cultures. Drug Metab. Dispos. **28**: 1270–1273. PMID:11038151.

Venkataramanan, R., Komoroski, B., and Strom, S. 2006. In vitro and in vivo assessment of herb drug interactions. Life Sci. **78**: 2105–2116. doi:10.1016/j.lfs.2005.12.021. PMID:16427092.

Wandel, C., Kim, R.B., Kajiji, S., Guengerich, P., Wilkinson, G.R., and Wood, A.J.J. 1999. P-glycoprotein and cytochrome P-450 3A Inhibition: dissociation of inhibitory potencies. Cancer Res. **59**: 3944–3948. PMID:10463589.

Zeng, X., and Zeng, X. 1999. Relationship between the clinical effects of berberine on severe congestive heart failure and its concentration in plasma studied by HPLC. Biomed. Chromatogr. **13**: 442–444. doi:10.1002/(SICI)1099-0801(199911)13:7<442::AID-BMC908>3.0.CO;2-A. PMID:10534753.

Zhou, S., Lim, L.Y., and Chowbay, B. 2004. Herbal modulation of p-glycoprotein. Drug Metab. Rev. **36**: 57–104. doi:10.1081/DMR-120028427. PMID:15072439.