Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 273 (2004) 135–147
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

The influence of enaminones on the transport and oral bioavailability of P-glycoprotein substrate therapeutic agents

Noha N. Salama [a], Kenneth R. Scott [b], Natalie D. Eddington [a,*]

[a] *Pharmacokinetics-Biopharmaceutics Laboratory, Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland at Baltimore, 20 Penn Street, Rm 543, Baltimore, MD 21201, USA*
[b] *Department of Pharmaceutical Sciences, College of Pharmacy, Howard University, Washington, DC 20059, USA*

Received 8 August 2003; received in revised form 22 December 2003; accepted 23 December 2003

Abstract

Objective: The enaminones have shown high P-gp affinity and may act as P-gp modulators. This study investigated the potential inhibition of the enaminones on paclitaxel efflux in vitro compared to cyclosporin A, a known P-gp inhibitor, and the effectiveness of select enaminones on paclitaxel oral absorption in rats. *Methods*: Caco-2 transport of [^{14}C]paclitaxel was evaluated in presence of various enaminones at 10^{-5} M. Concentration–effect (10^{-10} M to 10^{-4} M) profiles for the enaminones, DM27 and/or DM40, with paclitaxel and cyclosporin A were determined. Male Sprague–Dawley (250–275 g) rats were orally administered either [^{14}C]paclitaxel (30 μCi/kg) or [^{14}C]paclitaxel/DM27 (7 mg/kg) and blood samples were collected. Paclitaxel brain concentrations were measured. *Results*: $Papp_{(A-B)}$ of [^{14}C]paclitaxel, with DM27 and DM40 at 10^{-5} M, was significantly ($P < 0.05$) higher versus control. DM27 produced a 360% and a 139% increase in $Papp_{(A-B)Paclitaxel}$ and $Papp_{(A-B)Cyclosporin}$, respectively. DM40 displayed a 131% increase in $Papp_{(A-B)Paclitaxel}$ whereas cyclosporin A produced a 213% increase in $Papp_{(A-B)Paclitaxel}$. Rats in the DM27 group displayed large Vd_{ss}/F values (23.35 liters/kg versus 14.62 liters/kg) and lower AUC (5.47 μg/ml min versus 8.74 μg/ml min) versus control. However, significantly higher levels (2.25-fold) of paclitaxel were observed in the brains of the DM27 group. *Conclusion*: This study presents the enaminones as promising P-gp inhibitors with comparable potency to other P-gp inhibitors. Furthermore, the enaminones may improve conventional therapy when used in combination with P-gp substrate drugs.

Keywords: P-gp inhibitors; Enaminones; Paclitaxel; Oral bioavailability; Efflux transporters

1. Introduction

In order to optimize oral bioavailability, it is necessary to first identify the barriers to oral absorption. Multi-drug resistance (MDR), conferred by the active extrusion of drugs from the cell, is a phenomenon often seen in cancer cells that may become resistant to a wide spectrum of drugs with varying chemical structures or cellular targets (Gottesman and Pastan, 1993). In humans, the major protein products producing the MDR phenotype are the multidrug resistance gene product MDR1 P-glycoprotein (P-gp), the multidrug resistance associated proteins (MRP1–MRP6), the vesicular localized drug resistance related protein (LRP), and the breast cancer resistance proteins (BCRP) (Borst et al., 2000; Cole et al., 1992; Doyle et al., 1998; Juliano and Ling, 1976; Kool et al., 1996, 1999; Litman et al., 2001; Scheper et al., 1993; Stouch

* Corresponding author. Tel.: +1-410-706-6710; fax: +1-410-706-5017.
E-mail address: neddingt@rx.umaryland.edu (N.D. Eddington).

0378-5173/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2003.12.020

and Gudmundsson, 2002; Zaman et al., 1994). P-gp has been linked to incomplete and/or slow intestinal drug absorption of many drugs (Aungst, 1999; Suzuki and Sugiyama, 2000). It is a multifunctional protein that acts as an efflux pump for various hydrophobic compounds and regulates membrane composition via its flippase properties and ion transport (Roepe, 1995; Valverde et al., 1996). As of yet, it is not understood how P-gp recognizes and transports structurally diverse compounds. However, all transported compounds share some characteristics such as being hydrophobic or amphipathic in nature, containing a hydrophobic core and a polar or even charged domain (Schinkel, 1998). P-gp is expressed in the epithelial cells of the intestine, liver, adrenal glands, renal proximal tubule, placenta, testes, and hematopoietic cells and along the capillary endothelium of the blood brain barrier (BBB) (Deuchars and Ling, 1989).

An approach to increase the oral bioavailability of drugs is to inhibit their efflux by P-glycoprotein. MDR inhibitors (often referred to as modulators) are compounds, which alter P-gp function and are not themselves transported by P-gp (Martin et al., 2000). MDR modulators or inhibitors sensitize resistant cells and allow for drug entry into the cell or tissue (Ambudkar et al., 1999), thus improving the efficacy of conventional therapy when used in combination with P-gp substrate drugs. The three main mechanisms of modulators are: (1) direct interaction with one or more binding sites on P-gp, blocking transport by acting as competitive or non-competitive inhibitors; (2) inhibition of ATP binding, hydrolysis or coupling of ATP hydrolysis to the translocation of substrates; or (3) interaction with the lipid membrane of the cell thus perturbing the membrane environment or modifying the drug–membrane interaction (Ambudkar et al., 1999). Current P-gp inhibitors include; calcium channel blockers, calmodulin inhibitors, antiarrhythmics, antidepressants, immunomodulators, and antibiotics (Jetté et al., 1998). These P-gp inhibitors pose serious clinical risks because they may alter the pharmacokinetics of coadministered drugs in addition to possible side effects (verapamil cardiotoxicity, cyclosporin A nephrotoxicity) (Lum et al., 1993).

Enaminones are anticonvulsant compounds, which displayed P-gp substrate properties (Cox et al., 2001). Studies in bovine brain microvessel endothelial cells (BBMEC) displayed that the transport of enaminones exhibited high efflux ratios (Eddington et al., 2000). In vitro studies evaluating the transport and/or uptake of a series of enaminones alone and in the presence of P-gp inhibitors (e.g. verapamil and rhodamine123) in both BBMECs and *MCF-7/Adr* cell culture systems strongly suggested that P-gp may be responsible for enaminone efflux (Cox et al., 2001, 2002; Eddington et al., 2000). Further in vivo studies in P-gp-deficient mice demonstrated significantly higher (∼2–3-fold) enaminone levels in P-gp expressing tissues compared to wild-type mice (Cox et al., 2001, 2002). The use of enaminones to block the efflux of P-gp substrates may greatly increase oral bioavailability. The identification of substrates/inhibitors of a specific efflux transporter is conducted via several screening assays such as cytotoxicity assays, inhibition of efflux, transport experiments, and ATPase assay (Stouch and Gudmundsson, 2002). Cyclosporin A has been reported as a P-gp substrate (compounds that can be transported by P-gp) that may competitively inhibit the transport of coadministered P-gp substrates such as paclitaxel. In this study, screening of enaminones was performed by the inhibition of efflux approach, being a direct and reproducible functional assay for P-gp dependent transport. The effect of a series of enaminones on the transport of a P-gp substrate, paclitaxel, was investigated and compared to the effect of cyclosporin A. The effect of select enaminones on the transport of potent P-gp substrates such as cyclosporin A was also investigated. Paclitaxel was chosen as the P-gp substrate because it has a limited oral bioavailability mostly due to P-gp-mediated efflux despite susceptibility to cytochrome P450-mediated metabolism (Sparreboom et al., 1997; Woo et al., 2003). In addition, paclitaxel possesses significant therapeutic value as an antineoplastic agent for breast and ovarian cancer.

2. Materials and methods

2.1. Materials

The Caco-2 cell line was obtained from ATCC (Rockville, MD). Ketamine HCl injection, USP, was purchased from Bedford Laboratories (Bedford, OH). Xylazine was purchased from Sigma Chemical Co. (St Louis, MO). All chemicals were of analytical grade. All surgical supplies were purchased from World

Precision Instruments (Sarasota, FL). Experimental supplies and saline were purchased from Fisher Scientific (Fair Lawn, New Jersey). Polyethylene 50 (PE 50) tubing was obtained from Clay Adams (Parsippany, NJ). Paclitaxel-(2-benzoyl-ring-UL-^{14}C) (56.9 mCi/mmol, purity ≥95%) and [Mebmet-β-^{3}H]cyclosporin A (methyl-butenyl-methyl-threonine-β-^{3}H) (5–20 Ci/mmol) were purchased from Sigma and Amersham Radiochemicals (Piscataway, NJ), respectively. Ultima Gold and Universol Scintillation counting cocktails were purchased from Packard Bioscience (Meriden, CT) and ICN (Cost Mesa, CA), respectively. Solvable™ (Tissue and gel solubilizer, 0.5 M) was purchased from Packard Bioscience (Meriden, CT). Cell culture supplies (Dulbecco's modified Eagle medium, Dulbecco's phosphate buffer saline (PBS) (1×), non-essential amino acids, fetal bovine serum, L-glutamate, trypsin 0.25%–EDTA (1 mM), Penicillin G–streptomycin sulfate antibiotic mixture, gentamycin, and fungizone) were purchased from Gibco laboratories (Lenexa, KS). Transwell clusters, 12-well (0.4 μm pores, surface area 1 cm^2) were purchased from Corning Costar (Cambridge, MA). The enaminones were synthesized in the laboratory of Dr. Kenneth Scott at Howard University, Washington, DC. Table 1 provides the structural characteristics of the enaminones evaluated.

Jugular cannulated male Sprague–Dawley rats (250–275 g) were purchased from Harlan Laboratories (Indianapolis, Indiana) and housed individually in cages. Rats were fed rat chow and water "ad libitum" and maintained on a 12-h light/dark cycle. The jugular vein cannula was kept patent by flushing with heparinized physiological saline and heparinized glycerol. The protocol for the animal studies was approved by the School of Pharmacy, University of Maryland IACUC.

2.2. Methods

2.2.1. Caco-2 transport studies with enaminones

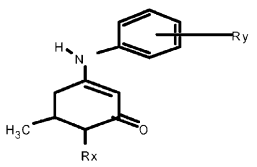
Caco-2 cell lines (passages 26–50) were seeded (100,000 cells/cm^2) on transwell costar filters (1 cm^2, 0.4 μm pore size) (Artursson, 1991; Hidalgo et al., 1989). The cells were grown for 21 days in Dulbecco's modified Eagle medium (1×), containing 1% penicillin–streptomycin, 2% glutamine, 10% fetal bovine serum, 1% non-essential amino acids, 0.2% gentamycin, and 0.4% fungizone, under standardized conditions (95% relative humidity, 5% CO_2, 37 °C) with medium change every other day. The cells were used between days 21 and 28. On the experiment day, the integrity of tight junctions was determined by measuring transepithelial electrical resistance (TEER) values using a Millicell®-ERS meter (Millipore Corp., Bedford, MA) and by the transport of a paracellular marker, mannitol. Batches of cells with TEER values >350 Ω cm^2, mannitol permeability $<2 \times 10^{-6}$ cm/s, and paclitaxel efflux ratios (B–A Papp/A–B Papp) within 10–20 were used.

For in vitro experiments, aqueous solutions of enaminones were prepared using dimethyl sulfoxide (DMSO) 0.4–7% and Pico-purified deionized water. Caco-2 cells were preincubated with PBS or each enaminone (E178, E121, LYN2, LYN3, K411, K421, DM1, 5, 10, 15, 16, 27, 32, 39, 40, 47, 49, 53, 54) at a concentration of 10^{-5} M for 45 min (Cox et al., 2001). After preincubation, a mixture of [^{14}C]paclitaxel (0.5 μCi/ml)/PBS (control) or [^{14}C]paclitaxel (0.5 μCi/ml)/enaminone (test) was applied to the apical chamber. Samples were collected from the receiver compartment at the following time points: 10, 20, 30, 45, 60, 75, 90, and 120 min and added to 5 ml universol scintillation counting cocktail. Radioactivity was measured using Beckman Coulter LS 6500 multi-purpose Scintillation counter.

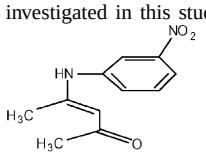
2.2.2. Concentration–effect studies with select enaminones

Based on the initial screening, DM27 and DM40 were found to display significant effects on paclitaxel transport. Caco-2 cells were preincubated with DM27 solution (7% DMSO) or DM40 solution (0.6% DMSO) at different concentrations: 10^{-10}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M for 45 min (Cox et al., 2001). After preincubation, a mixture of [^{14}C]paclitaxel (0.5 μCi/ml)/PBS or [^{14}C]paclitaxel/DM27 or DM40 was applied to the apical chamber. Samples were collected from the receiver compartment at 10, 20, 30, 45, 60, 75, 90, and 120 min. Samples were added to 5 ml universol scintillation cocktail. Radioactivity was measured by Beckman Coulter LS 6500 multi-purpose Scintillation counter. Similar in vitro studies were performed with [^{3}H]cyclosporin A/DM27 (at concentration range; 10^{-10} to 10^{-4} M) versus [^{3}H]cyclosporin A alone. The inhibitory effect

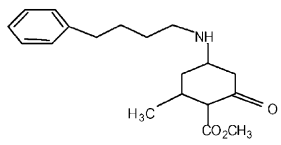
Table 1
The structural characteristics of the enaminones investigated in this study



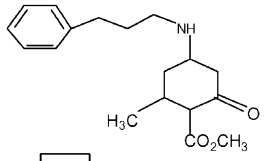
General structure of enaminones



A



B



C

Compound[a]	Molecular weight	*C* log *P*[b]	Substitution groups	
			R_x	R_y
LYN2	380.31	4.62	$CO_2C(CH_3)_3$	4′-Br
K421	246.26	2.75	H	4′-NO_2
DM1	320.39	2.92	$CONHC_6H_5$	H
DM27	234.73	3.37	H	4′-Cl
DM5	293.75	3.23	CO_2CH_3	4′-Cl
DM54	293.75	2.93	CO_2CH_3	2′-Cl
E121	307.10	3.76	$CO_2C_2H_5$	4′-Cl
DM10	275.33	1.55	CO_2CH_3	3′-OH
DM15	275.33	1.55	CO_2CH_3	4′-OH
DM16	302.36	1.08	CO_2CH_3	4′-$CONH_2$
DM32	220.22	2.08[c]	A	
DM39	315.41	3.99	B	
DM40	303.44	3.46	C	
DM47	327.32	3.33	CO_2CH_3	3′-CF_3
K411	269.26	3.47	H	4′-CF_3
DM49	343.30	2.88	CO_2CH_3	3′-OCF_3
LYN3	385.42	4.12	$CO_2C(CH_3)_3$	4′-OCF_3
DM53	338.42	0.76	CO_2CH_3	4′-SO_2NH_2
E178	352.41	0.99	$CO_2C_2H_5$	3′-SO_2NH_2

[a] Chemical names are: LYN2: *tert*-butyl-4-(4-bromophenylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate; K421: 5-methyl-3-(4-nitrophenylamino)-cyclohex-2-enone; DM1: 6-methyl-2-oxo-4-phenylaminocyclohex-3-ene carboxylic acid phenylamide; DM27: 3-(4-chlorophenylamino)-5-methylcyclohex-2-enone; DM5: methyl-4-(4-chlorophenylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate; DM54: methyl-4-(2-chlorophenylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate; E121: ethyl-4-(4-chlorophenylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate; DM10: methyl-4-(3-hydroxyphenylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate; DM15: methyl-4-(4-hydroxyphenylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate; DM16: methyl-4-(4-carbamoylphenylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate; DM32: 4-(3-nitrophenylamino)-pent-3-en-2-one; DM39: methyl-4-(4-phenylbutylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate; DM40: methyl-4-(3-phenylpropylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate; DM47: methyl-4-(3-trifluoromethylphenyl-amino)-6-methyl-2-oxocyclohex-3-ene carboxylate; K411: 3-(4-trifluoromethylphenyl-amino)-5-methylcyclohex-2-enone; DM49: methyl-4-(3-trifluoromethylphenylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate; LYN3: *tert*-butyl-4-(4-trifluoromethoxy-phenylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate; DM53: methyl-4-(4-sulfamoylphenylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate; E178: ethyl-4-(3-sulfamoylphenylamino)-6-methyl-2-oxocyclohex-3-ene carboxylate.

[b] Determined by reference # (MacLogP Program, Version 4.0 BioByte Corp., Claremont, CA, 91711, USA).

[c] Determined by ACDlab software.

of cyclosporin A (5×10^{-6} M) (Choo et al., 2000; Dintaman and Silverman, 1999; Naruhashi et al., 2001; van der Sandt et al., 2000) on paclitaxel transport was investigated by incubation of the cells with cyclosporin A followed by transport studies for [^{14}C]paclitaxel in the presence of cyclosporin A.

2.2.3. Oral absorption studies after intraduodenal dosing in rats

Jugular vein cannulated Sprague–Dawley rats were anaesthetized with intraperitoneal injection of a ketamine/xylazine solution (80 mg/kg ketamine, 12 mg/kg xylazine). PE 50 tubing was used for

duodenal cannulation according to the method reported by Lukas and Moreton (1979). Cannulas were tailored to the same length and were inserted at approximately the same site in the duodenum. The cannula was anchored and exteriorized at the neck. PBS was infused very slowly to ensure the free flow of fluids through the intraduodenal cannula. Rats were allowed to recover from the surgery for two days prior to the initiation of the study.

For the in vivo experiments, DM27 was solubilized in a mixture composed of 20% alcohol, 20% DMSO, 20% propylene glycol, and 40% water. The animals were fasted for >12 h prior to the experiment, with free access to water. Rats ($n = 3$–4 per group) were randomly assigned to receive intraduodenaly: (1) [^{14}C]paclitaxel (30 μCi/kg)/solvent mixture, (2) [^{14}C]paclitaxel (30 μCi/kg)/DM27 (7 mg/kg). DM27 (in test) and the solvent mixture (in control) were administered 5 min prior to [^{14}C]paclitaxel administration. The solution volume administered was adjusted to 2 ml with physiological saline. Blood samples were collected via the jugular cannula at: 10, 20, 30, 45, 60, 90, 120, 240, 360, and 480 min. Blood samples were centrifuged (13,000 rpm for 10 min) and plasma was obtained and immediately measured. Scintillation cocktail (5 ml) was added and radioactivity was measured by Beckman Coulter LS 6500 multi-purpose Scintillation counter. At the end of the experiment, rats were sacrificed by CO_2 asphyxiation and the duodenum was inspected in order to ensure that the intraduodenal cannula was in place during dosing. In order to evaluate the amount of paclitaxel distributed into the brain tissue and the effect of oral P-gp inhibition on BBB, brains were excised at 480 min, stored at −70 °C and subsequently analyzed for paclitaxel.

2.2.4. Quantitation of radiolabelled paclitaxel in rat brains

Brains were weighed and homogenized with an equal volume of PBS. Solvable (1 ml) tissue solubilizer was added to the brain homogenate (200 μl) then incubated at 50 °C for 3 h. Glacial acetic acid (50 μl) was added to the homogenate and heated at 50 °C for 1 h for decolorization then left to cool to room temperature. Ultima Gold scintillation cocktail (10 ml) was added to the homogenate and kept at room temp for 1 h before counting by Beckman Coulter LS 6500 multi-purpose Scintillation counter. Blank brains were treated in the same way for baseline reference. A certain amount (100 μl) of stock [^{14}C]paclitaxel solution was added to some blank brain homogenate and treated similarly for standards.

2.3. Data analysis

Apparent permeability coefficients (P_{app}) were calculated by

$$P_{app} = \frac{dQ/dt}{A \cdot C_o} \quad (1)$$

where dQ/dt is the linear appearance rate of mass in the receiver solution (dpm/min), A is the cross-sectional area (1 cm^2), C_o is the initial concentration in the donor compartment (dpm/ml). All values are represented as mean and standard error of the mean (S.E.M.). The percent fold increase (percent enhancement ratio) in transport, R, was calculated by

$$R = \frac{\text{Papp}_{\text{A–B (+Enaminone or Cyclosporin A)}}}{\text{Papp}_{\text{A–B (Control)}}} \times 100 \quad (2)$$

Data obtained after the in vivo administration of DM27 were analyzed using WinnonLin^{TM} (Pharsight Inc., Mountain View, CA). The amount of radiolabelled drug absorbed was converted to concentrations using the specific activity of the radiolabelled stock drug solution. The pharmacokinetic parameters were calculated with non-compartmental analysis based on the statistical moment theory. The area under the plasma concentration–time curve (AUC) was calculated using the linear trapezoidal rule. The highest observable concentration and associated time point were defined as maximum concentration (C_{max}) and corresponding time (T_{max}), respectively. Data are presented as mean ± S.E.M. The percent fold increase, R, for the pharmacokinetic parameters (PK) was calculated from the following equation:

$$R = \frac{\text{PK parameter}_{(+\text{DM27})}}{\text{PK parameter}_{(\text{control})}} \times 100 \quad (3)$$

In order to evaluate the influence of oral P-gp inhibitor administration on the BBB efflux of paclitaxel, the amount of radiolabelled paclitaxel detected at the end of the experiment in the brains of paclitaxel/DM27 group was compared to the control group. Radiolabelled drug concentration in the brain was calculated

based on the specific activity and concentration of the stock solution in reference to the readings of a group of standards. The standards were prepared from adding specific known amounts of stock paclitaxel to blank brain tissues. Permeability coefficients, pharmacokinetic parameters, and brain amounts were statistically compared by Student's *t*-test or ANOVA using Dunnett's post hoc analysis at $P < 0.05$.

3. Results

3.1. Caco-2 transport studies with enaminones

Apical to basolateral (A–B) permeability of paclitaxel alone and in the presence of each investigated enaminone was determined across Caco-2 cells. The cells were preincubated with E178, E121, LYN2, LYN3, K411, K421, DM1, 5, 10, 15, 16, 27, 32, 39, 40, 47, 49, 53, 54 for 45 min before adding [^{14}C]paclitaxel (0.5 μCi/ml) apically. Table 2 lists the percent enhancement ratios for paclitaxel Papp with various enaminones. The percent fold increase in paclitaxel $Papp_{A–B}$ was statistically significant ($P < 0.05$) with DM27 and DM40 displaying 143.7 and 124.3% fold, respectively, compared to the control.

Table 2
The percent enhancement ratios (±S.E.M.) for paclitaxel $Papp_{(A–B)}$ (0.5 μCi/ml) in the presence of various enaminone compounds (10^{-5} M) relative to control paclitaxel $Papp_{(A–B)}$ across Caco-2 cells

Compound	Percent enhancement ratio (±S.E.M.)
E178	87.88 (2.87)
E121	94.16 (3.64)
LYN3	112.33 (2.08)
LYN2	77.59 (1.89)
K411	111.82 (15.18)
K421	78.1 (6.33)
DM1	114.08 (1.73)
DM5	91.91 (9.88)
DM10	84.92 (2.73)
DM15	85.77 (0.82)
DM16	104.35 (3.35)
DM27	143.65 (2.73)*
DM32	111.99 (8.19)
DM39	110.93 (10.03)
DM40	124.32 (7.35)*
DM47	117.85 (9.23)
DM49	118.04 (2.09)
DM53	83.52 (1.62)
DM54	97.04 (5.44)

The efflux ratios for paclitaxel across Caco-2 cells ranged between 10 and 20. *Note*. $Papp_{(A–B)}$ for paclitaxel: 5.7 (±0.6) × 10^{-6} cm/s ($n = 3$).
* Significant at $P < 0.05$ compared to paclitaxel alone.

The concentration–effect profiles for paclitaxel $Papp_{A–B}$ with DM27 and DM40 were investigated at different concentrations: 10^{-10} to 10^{-4} M. DM27 and DM40 profiles displayed a bell shape pattern. Incubation of DM27 (10^{-10} M) solubilized in DMSO (7%) did not lead to any statistically significant alterations in paclitaxel P_{app} values. This observation proves that neither DM27 at this low concentration nor DMSO (7%) affected P-gp functionality. This observation confirms earlier reports which validated the use of DMSO as a solubilizing agent for relatively insoluble compounds in Caco-2 cell experiments and emphasized the absence of DMSO effect on P-gp modulation or on Caco-2 monolayer barrier functions (Booth et al., 1998; Da Violante et al., 2002; Dorr and Liddil, 1991; Krishna et al., 2001; Stammati et al., 1997; Zhou et al., 1993).

DM27 showed statistically significant ($P < 0.05$) increase in paclitaxel $Papp_{A–B}$ at each concentration except 10^{-10} M (Fig. 1A) while DM40 displayed statistically significant enhancement at 10^{-8} and 10^{-7} M (Fig. 1B). Based on the promising results with DM27 and paclitaxel, another P-gp substrate, cyclosporin A, was evaluated with DM27. Studies with cyclosporin A/DM27 displayed statistically significant ($P < 0.05$) increase in cyclosporin A $Papp_{A–B}$ at 10^{-7} M. The profile for cyclosporin A/DM27 (Fig. 2) followed the same bell shape pattern displayed with paclitaxel (Fig. 1A).

In order to compare the inhibitory effect of DM27 on paclitaxel efflux with other P-gp inhibitors, transport of [^{14}C]paclitaxel in the presence of cyclosporin A (5×10^{-6} M), a common P-gp inhibitor, was performed. Cyclosporin A displayed 212.6% fold increase in paclitaxel $Papp_{(A–B)}$ which is comparable to the enhancement displayed with DM27 at 10^{-5} M on paclitaxel transport yet less than the enhancement effect at 10^{-6} M.

3.2. Oral absorption studies after intraduodenal dosing in rats

The in vitro Caco-2 studies displayed that DM27 was able to most successfully minimize P-gp efflux

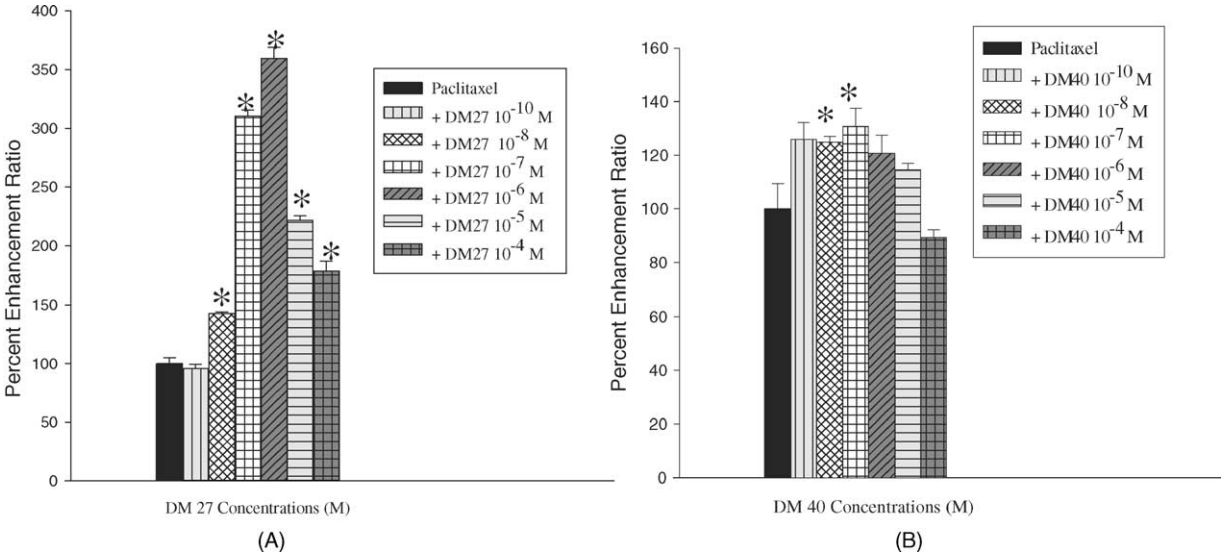


Fig. 1. (A) The percent enhancement ratios (±S.E.M.) for the transport of paclitaxel in the presence of DM27 at 10^{-10}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M across Caco-2 cells ($n = 3$–4). *Significant at $P < 0.05$ compared to paclitaxel alone. (B) The percent enhancement ratios (±S.E.M.) for the transport of paclitaxel in the presence of DM40 at 10^{-10}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M across Caco-2 cells ($n = 3$–4). *Significant at $P < 0.05$ compared to paclitaxel alone.

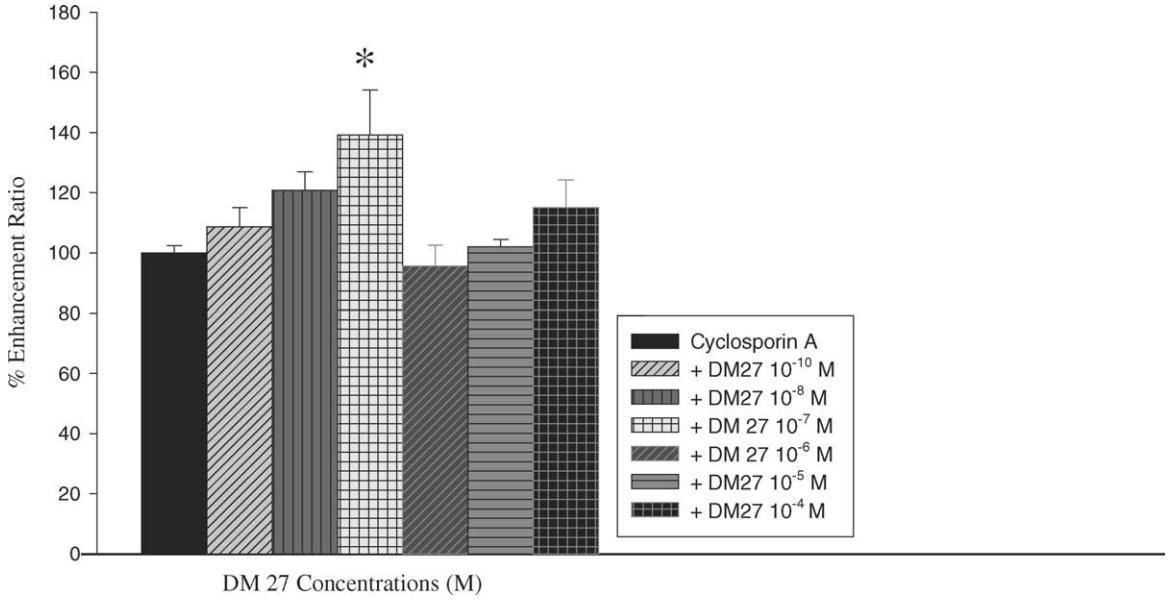


Fig. 2. The percent enhancement ratios (±S.E.M.) for the transport of cyclosporin A in the presence of DM27 at 10^{-10}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M across Caco-2 cells ($n = 3$–4). *Significant at $P < 0.05$ compared to cyclosporin A alone.

and in vivo studies were carried out with this enaminone. Male Sprague–Dawley rats cannulated in the jugular vein and duodenum randomly received [^{14}C]paclitaxel or [^{14}C]paclitaxel/DM27 (7 mg/kg). Table 3 depicts the mean (±S.E.M.) pharmacokinetic parameters determined by non-compartmental analysis. DM27-treated group displayed greater Vd_{ss}/F (23.35 liters/kg versus 14.62 liters/kg), larger Cl/*F*

Table 3
The mean (±S.E.M.) pharmacokinetic parameters in male Sprague–Dawley rats after ID administration of [^{14}C]paclitaxel (30 μCi/kg)/solvent mixture and [^{14}C]paclitaxel (30 μCi/kg)/DM27 (7 mg/kg)

Treatment	[^{14}C]paclitaxel	[^{14}C]paclitaxel + DM27	Ratio (test/control)
C_{max} (μg/ml)	0.06 (0.004)*	0.03 (0.004)	0.5
T_{max} (min)	14.67 (2.67)	17.3 (±2.67)	1.18
AUC (μg/ml min)	8.74 (0.58)*	5.47 (0.48)	0.63
Vd_{ss}/F (liters/kg)	14.62 (4.23)*	23.35 (1.00)	1.6
Cl/F (liters/min/kg)	0.04 (0.00)*	0.05 (0.00)	1.25
$t_{1/2}$ (h)	4.64 (1.43)	5.44 (0.31)	1.17

* Significant at $P < 0.05$ compared to paclitaxel alone.

(0.05 liters/min/kg versus 0.04 liters/min/kg) and longer $t_{1/2}$ (5.44 h versus 4.64 h). The differences in Cl/F and Vd_{ss}/F obtained after [^{14}C]paclitaxel/DM27 and [^{14}C]paclitaxel administration, respectively, were statistically significant ($P < 0.05$) (Fig. 3). The control group displayed two-fold higher C_{max} and 1.6-fold higher AUC compared to test.

In addition to evaluating the plasma concentration versus time profiles, the distribution of paclitaxel into the brain was examined, since it also expresses P-gp apically (Egan and Lauri, 2002). The amount of paclitaxel determined in the brains of the test group was significantly ($P < 0.05$) 2.25-fold greater than the control group (5.023 ng/g versus 2.23 ng/g,

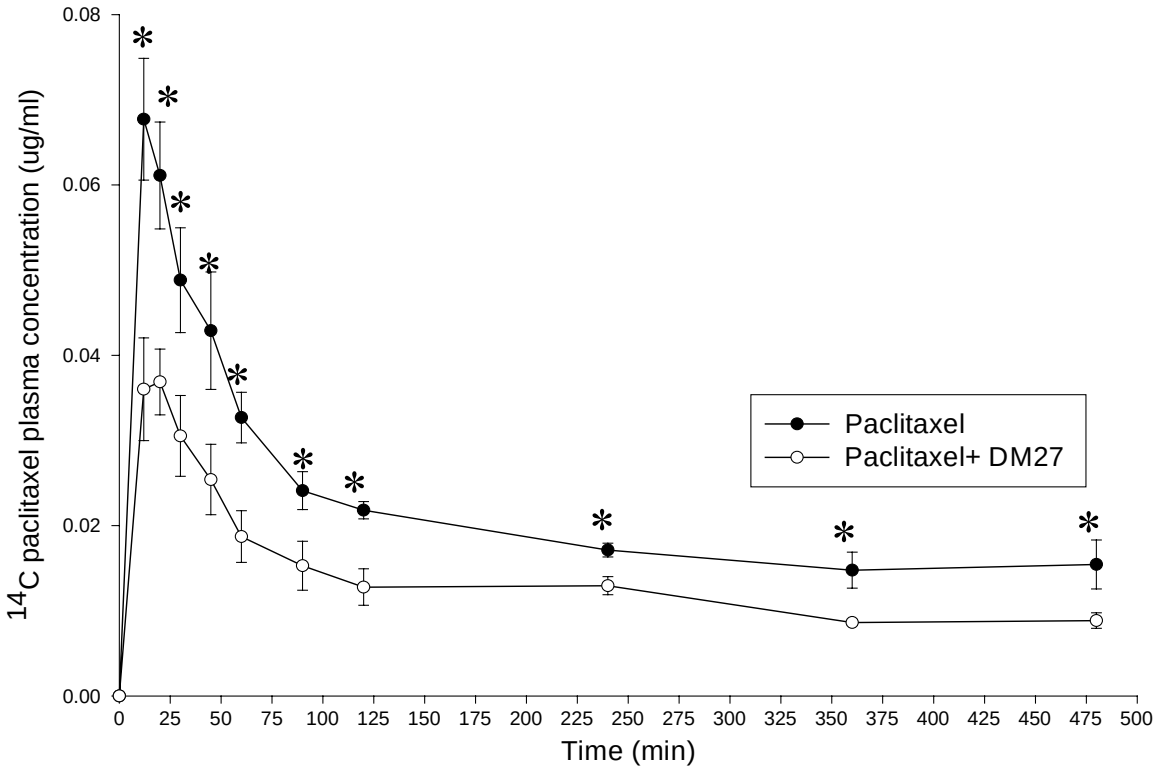


Fig. 3. The mean (±S.E.M.) plasma concentration–time profiles for [^{14}C]paclitaxel after control (●) or DM27 (○) intraduodenal dosing to male Sprague–Dawley rats ($n = 3$–4 per group). ∗Significant at $P < 0.05$.

respectively). The brain to plasma ratio after 8 h was 2 and 7.8 for the control and test groups, respectively.

The use of radiolabelled drugs in research has always raised doubts about the contribution of metabolites in the measurements. However, paclitaxel is mainly metabolized by hepatic and biliary metabolism and mainly excreted in the bile (Monsarrat et al., 1990) with 90–98% radioactivity recovered in feces (Sparreboom et al., 1997; Gaver et al., 1993). In vivo metabolism studies with paclitaxel in male Sprague–Dawley rats identified two metabolites in the liver, but none in the plasma or other tissues (Klecker et al., 1994). Major fractions of dose excreted in the form of parent drug or hydroxylated metabolites (Sparreboom et al., 1998). Hydroxylated metabolites were not detectable in plasma or any tissues, except liver and tissues of the GIT, which indicates that the major part of these compounds is secreted directly into the bile and that reabsorption from the intestinal lumen is low (Sparreboom et al., 1996). Based on these earlier studies, it is highly likely that the radiolabelled paclitaxel quantitated in the brain represents the parent compound.

4. Discussion

Enaminones are beta dicarbonyl enamines, which displayed P-gp affinity with efflux ratios ranging from 1.86 to 5.3 (Eddington et al., 2000). The structure of enaminones is consistent with the general characteristics of P-gp reversal agents including high lipophilicity ($C\log P$ ranges from ~2.5 to 3.88) and a secondary amine group. In addition, their structure allows for potential conformational changes into a phenothiazine structure (Fig. 4A) that is associated with known P-gp reversal agents (Ambudkar et al., 1999). Based on these observations, we decided to investigate the effect of various enaminones on paclitaxel efflux in search of potent P-gp inhibitors (Table 1).

In this study, DM27 and DM40 significantly increased paclitaxel transepithelial transport by 143.7 and 124.3%, respectively, at 10^{-5} M in comparison to paclitaxel transport in their absence. The P-gp inhibitory effect of DM27 (at 10^{-7} M) was also shown with cyclosporin A, a P-gp substrate and inhibitor, with a 139.2% fold increase in cyclosporin A transport across Caco-2 cells. Cyclosporin A has been used as a P-gp inhibitor to minimize paclitaxel efflux. The effect of cyclosporin A, a P-gp inhibitor, was compared to DM27 effect. At 10^{-6} M, DM27 was capable of increasing paclitaxel Papp by 360% fold whereas cyclosporin A, at 5×10^{-6} M, increased paclitaxel Papp by ~213%. This comparison demonstrates that the enaminones are promising P-gp inhibitors with comparable/better effects than current P-gp inhibitors. In a previous study, Seelig and co-workers suggested that substrate recognition patterns for human P-gp consists of two electron donor groups with a spatial separation of 2.5 ± 0.3 Å (type I units) or three electron donor groups with a spatial separation of the two outer groups of 4.6 ± 0.6 Å (type II units[1]) (Fig. 4B) (Seelig, 1998) and that lipophilicity is a modulator of the substrate–transporter interaction. Based on this theory, DM27 and DM40 are in excellent agreement with the bond lengths and lipophilicity with A–B of 2.573 and 2.575 Å and A–C of 5.691 and 5.621 Å for DM27 and DM40, respectively.

Some of the investigated enaminones have already been reported to have high efflux ratios (e.g. DM5) yet failed to increase the A–B transport of paclitaxel in this study (Cox et al., 2001). This is probably due to having lower affinity or substrate specificity to P-gp compared to paclitaxel. It is also possible that the concentration investigated was too low to show a significant inhibitory effect. DM27 is structurally similar to DM5 except for the 4-carbomethoxy group in DM5 (Table 1). Yet, DM27 acted as a strong P-gp inhibitor to paclitaxel efflux while DM5 did not. Thus, it would appear that the 4′-Cl substitution might be important for the P-gp recognition site and the appropriate alignment (in both DM5 and DM27) yet the ester function at C1 decreases the specificity or affinity of the enaminone to P-gp compared to paclitaxel thus resulting in no effect on paclitaxel efflux in the case of DM5. Similar behavior was seen in a previous study where DM49 was reported to have lesser substrate specificity than rhodamine123 (R123) [commonly used to evaluate P-gp functional activity (Fontaine et al., 1996)] despite possessing high efflux ratio across BBMECs, thus not affecting R123 uptake in vitro (Cox et al., 2001). Earlier reports and our studies show that

[1] Seelig describes the electron donor unit: “A” as strong (oxygen-containing) hydrogen bond acceptor or electron donor groups.

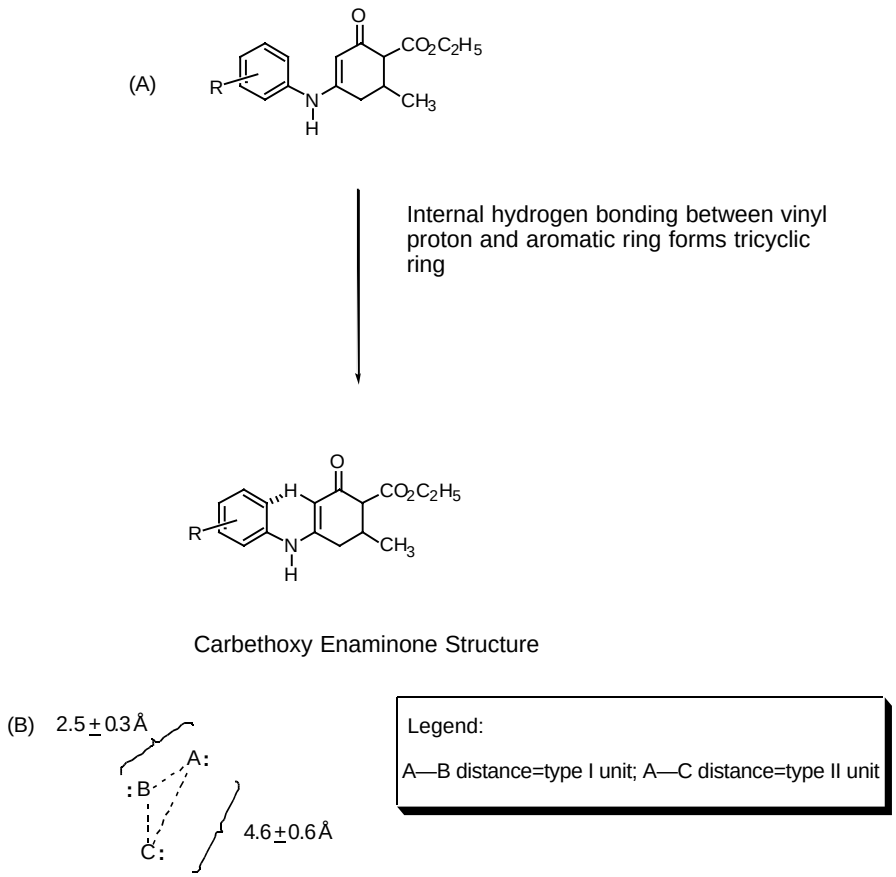


Fig. 4. (A) Conformational changes in the carboethoxy enaminone structure lead to the formation of a phenothiazine ring. (B) Seelig hypothesis for substrate recognition for human P-gp encoded by MDR1.

categorizing a compound as a P-gp inhibitor depends on the P-gp substrate under investigation. The P-gp inhibitors; enaminones, reported in this study were referenced to paclitaxel, a more specific substrate to P-gp and could be used as a P-gp inhibitor to R123 efflux.

As seen clearly in the DM27 and DM40 concentration–effect profiles, the enaminones act as P-gp inhibitors in a concentration-dependent manner exhibiting bell shape profiles with minimal inhibitory effects at higher concentrations. This pattern was observed with pluronic block co-polymers on R123 uptake (Batrakova et al., 2001) and similar bell shape profiles for the drug concentration against ATPase activity indicated the presence of two binding sites (high and low affinity) for the drug (Litman et al., 1997). Investigation of the exact mechanism of P-gp inhibition by DM27 and DM40 will clarify the reasons for the bell-shape pattern. Predictions of structure–affinity relationships could promote the rational design of drugs/prodrugs targeting specific transporters.

In addition to the in vitro evaluation of the enaminones, we examined their influence on the oral bioavailability of a known P-gp substrate, paclitaxel. It should be noted that the enaminones have been developed as anticonvulsants and as such, doses used were not therapeutically active (DM27 ED_{50} = 14.72 mg/kg versus 7 mg/kg ID dose) (Eddington et al., 2000; Scott et al., 1995). It was expected that P-gp inhibition in the GI tract by DM27 would result in an increase in oral absorption of paclitaxel. However in our studies, the plasma paclitaxel concentrations were significantly higher with C_{max} two-fold

and AUC 1.6-fold higher for the control group. Similar findings have been reported for the administration of cyclosporin A with the P-gp inhibitor, quercetin, when given orally to both pigs and rats (Hsiu et al., 2002). In those studies, quercetin decreased the oral bioavailability of cyclosporin A. Recent studies by Matheny and co-workers reported decreased oral bioavailability of digoxin in mice and humans with increased brain distribution due to concomitant P-gp induction and inhibition by rifampin (Matheny et al., 2003). Although we did not observe an enhanced oral absorption of paclitaxel with DM27, the P-gp modulating effects of this enaminone were apparent in our evaluation of the brain tissue distribution. P-gp has been reported to be expressed in brain capillary endothelial cells (Batrakova et al., 2001). Paclitaxel brain levels were 2.25-fold higher in the DM27-treated group. In addition, a larger Vd_{ss}/F was observed for the DM27 group, as such, differences in AUC may be due to the modulation of P-gp via DM27. This of course is predicated on the bioavailabilities (F) being similar in both groups (e.g. no plasma protein binding displacement of paclitaxel by DM27).

As previously stated, after oral dosing, DM27 appeared to modulate the distribution of paclitaxel in the brain, without a similar effect on plasma levels. DM27 possesses favorable characteristics [low MW (235), high lipophilicity ($C \log P = 3.37$)] for fast absorption through the lipid bilayer of the gastrointestinal tract (Egan and Lauri, 2002). This potential fast absorption may have limited its effectiveness on modulating P-gp in the GI tract. Thus, it is possible that by the time paclitaxel was administered (5 min after DM27 administration), a significant amount of DM27 had already been absorbed and distributed. Nonetheless, DM27 was able to cross the BBB easily where it interacted with P-gp inhibiting paclitaxel efflux at the BBB leading to a significant increase in the amount of paclitaxel distributed into the brain.

In summary, this study displayed the effectiveness of the enaminones in enhancing in vitro permeability and in vivo brain delivery of paclitaxel, a drug displaying high P-gp efflux and low oral bioavailability. The enaminone enhancing effects observed in vitro were significantly higher than our controls. The extent of transport enhancement was comparable/higher than common P-gp inhibitors such as cyclosporin A. The in vivo oral absorption study did not show an increase in the plasma drug levels with DM27, but illustrated the potential usefulness of the enaminones in enhancing drug delivery to the brain. Structural modifications for the enaminones may provide ‘ideal’ modulators, capable of pharmacologically modulating P-gp function, enhancing drug bioavailability and therapeutic effectiveness.

Acknowledgements

This research was supported by a grant from the National Institutes of Health (1 R21 GM63494-01), for which K.R.S. is the principal investigator and a grant from the National Cancer Institute (CA87564-01A1).

References

Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramachandra, M., Pastan, I., Gottesman, M.M., 1999. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Ann. Rev. Pharmacol. Toxicol. 39, 361–398.

Artursson, P., 1991. Cell cultures as models for drug absorption across the intestinal mucosa. Crit. Rev. Ther. Drug Carr. Syst. 8, 305–330.

Aungst, B.J., 1999. P-glycoprotein, secretory transport, and other barriers to the oral delivery of anti-HIV drugs. Adv. Drug Deliv. Rev. 39, 105–116.

Batrakova, E.V., Li, S., Vinogradov, S.V., Alakhov, V.Y., Miller, D.W., Kabanov, A.V., 2001. Mechanism of pluronic effect on P-glycoprotein efflux system in blood–brain barrier: contributions of energy depletion and membrane fluidization. J. Pharmacol. Exp. Ther. 299, 483–493.

Booth, C.L., Brouwer, K.R., Brouwer, K.L., 1998. Effect of multidrug resistance modulators on the hepatobiliary disposition of doxorubicin in isolated perfused rat liver. Cancer Res. 58, 3641–3648.

Borst, P., Evers, R., Kool, M., Wijnholds, J., 2000. A family of drug transporters: the multidrug resistance-associated proteins. J. Natl. Cancer Inst. 92, 1295–1302.

Choo, E.F., Leake, B., Wandel, C.H.I., Wood, A.J., Wilkinson, G.R., Kim, R.B., 2000. Pharmacological inhibition of P-glycoprotein transport enhances the distribution of HIV-1 protease inhibitors into brain and testes. Drug Metab. Dispos. 28, 655–660.

Cole, S.P.C., Bhardwaj, G., Gerlach, J.H., Mackie, J.E., Grant, C.E., Almquist, K.C., Stewart, A.J., Kurz, E.U., Duncan, A.M.V., Deeley, R.G., 1992. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 258, 1650–1654.

Cox, D.S., Scott, K.R., Gao, H., Raje, S., Eddington, N.D., 2001. Influence of multidrug resistance (MDR) proteins at the blood–brain barrier on the transport and brain distribution of enaminone anticonvulsants. J. Pharm. Sci. 90, 1540–1552.

Cox, D.S., Scott, K.R., Gao, H., Eddington, N.D., 2002. Effect of P-glycoprotein on the pharmacokinetics and tissue distribution of enaminone anticonvulsants: analysis by population and physiological approaches. J. Pharmacol. Exp. Ther. 302, 1096–1104.

Da Violante, G., Zerrouk, N., Richard, I., 2002. Evaluation of the cytotoxicity effect of dimethyl sulfoxide (DMSO) on Caco-2/TC7 colon tumor cell cultures. Biol. Pharm. Bull. 25, 1600–1603.

Deuchars, K., Ling, V., 1989. P-glycoprotein and multidrug resistance in cancer chemotherapy. Semin. Oncol. 16, 156–165.

Dintaman, J.M., Silverman, J.A., 1999. Inhibition of P-glycoprotein by D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS). Pharm. Res. 16, 1550–1556.

Dorr, R.T., Liddil, J.D., 1991. Modulation of mitomycin C-induced multidrug resistance in vitro. Cancer Chemother. Pharmacol. 27, 290–294.

Doyle, L.A., Yang, W., Abruzzo, L.V., Krogmann, T., Gao, Y., Rishi, A.K., Ross, D.D., 1998. A multidrug resistance transporter from human MCF-7 breast cancer cell. Proc. Natl. Acad. Sci. U.S.A. 95, 15665–15670.

Eddington, N.D., Cox, D.S., Roberts, R.R., Stables, J.P., Powell, C.B., Scott, K.R., 2000. Enaminones-versatile therapeutic pharmacophores. Further advances. Curr. Med. Chem. 7, 417–536.

Egan, W.J., Lauri, G., 2002. Prediction of intestinal permeability. Adv. Drug Deliv. Rev. 54, 273–289.

Fontaine, M., Elmquist, M.F., Miller, D.W., 1996. Use of rhodamine 123 to examine the functional activity of P-glycoprotein in primary cultured brain microvessel endothelial cell monolayers. Life Sci. 59, 1521–1531.

Gaver, R.C., Deeb, G., Willy, T.A., et al., 1993. The disposition of paclitaxel (taxol) in the rat. Proc. Am. Assoc. Cancer Res. 34, 390 (abstract).

Gottesman, M.M., Pastan, I., 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu. Rev. Biochem. 62, 385–427.

Hidalgo, I.J., Raub, T.J., Borchardt, R.T., 1989. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 96, 736–749.

Hsiu, S.L., Hou, Y.C., Wang, Y.H., Tsao, C.W., Su, S.F., Chao, P.D., 2002. Quercetin significantly decreased cyclosporine oral bioavailability in pigs and rats. Life Sci. 72, 227–235.

Jetté, L., Murphy, G.F., Béliveau, R., 1998. Drug binding to P-glycoprotein is inhibited in normal tissues following SDZ-PSC 833 treatment. Int. J. Cancer 76, 729–737.

Juliano, R.L., Ling, V., 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochem. Biophys. Acta 455, 152–162.

Klecker, R.W., James-Dow, C.A., Egorin, M.J., Erkmen, K., Parker, R.J., Stevens, R., Collins, J.M., 1994. Effect of cimetidine, probenecid and ketoconazole on the distribution, biliary secretion and metabolism of [^{3}H]Taxol in the Sprague Dawley rat. Drug Metab. Dispos. 22, 254–258.

Kool, M., de Haas, M., Scheffer, G.L., Scheper, R.J., Van Eijk, M.J., Juijn, J.A., Baas, F., Borst, P., 1996. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res. 57, 3537–3547.

Kool, M., Van der Linden, M., de Haas, M., Scheffer, G.L., de Vree, J.M., Smith, A.J., Jansen, G., Peters, G.J., Ponne, N., Scheper, R.J., Elferink, R.P., Baas, F., Borst, P., 1999. MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc. Natl. Acad. Sci. U.S.A. 96, 6914–6919.

Krishna, G., Chen, K.J., Lin, C.C., Nomeir, A.A., 2001. Permeability of lipophilic compounds in drug discovery using in vitro human absorption model, Caco-2. Int. J. Pharm. 222, 77–89.

Litman, T., Zeuthen, T., Skovsgaard, T., Stein, W.D., 1997. Structure–activity relationships of P-glycoprotein interacting drugs: kinetic characterization of their effects on ATPase activity. Biochem. Biophys. Acta 1361, 159–168.

Litman, T., Druley, T.E., Stein, W.D., Bates, S.E., 2001. From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. CMLS, Cell. Mol. Life Sci. 58, 931–959.

Lukas, S., Moreton, J., 1979. A technique for chronic intragastric drug administration in the rat. Life Sci. 25, 593–600.

Lum, B.L., Gosland, M.P., Kaubisch, S., Sikic, B.I., 1993. Molecular targets in oncology: implications of the multidrug resistance gene. Pharmacotherapy 13, 88–109.

Martin, C., Berridge, G., Mistry, P., Charlton, P., Callaghan, R., 2000. Communication between multiple drug binding sites on P-glycoprotein. Mol. Pharmacol. 58, 624–632.

Matheny, C.J., Ali, R.Y., Yang, X., Pollack, G.M., 2003. Complex interaction of rifampin and digoxin: evidence for concomitant P-glycoprotein induction and inhibition. In: Proceedings of the 18th Annual Meeting and Exposition of the American Association of Pharmaceutical Scientists, October 26–30, Salt Lake City, Utah (abstract).

Monsarrat, B., Mariel, E., Cros, S., et al., 1990. Taxol metabolism. Isolation and identification of three major metabolites of taxol in rat bile. Drug Metab. Dispos. 18, 896–901.

Naruhashi, K., Tamai, I., Inoe, N., Muraoka, H., Sai, Y., Suzuki, N., Tsuji, A., 2001. Active intestinal secretion of new quinolone antimicrobials and the partial contribution of P-glycoprotein. J. Pharm. Pharmacol. 53, 699–709.

Roepe, P.D., 1995. The role of the MDR protein in altered drug translocation across cell membranes. Biochem. Biophys. Acta 1241, 385–406.

Scheper, R.J., Broxterman, H.J., Scheffer, G.L., Kaaijk, P., Dalton, W.S., Van Heijningen, T.H.M., van Kalken, C.K., Slovak, M.L., de Vries, E.G.E., van der Valk, P., Meijer, C.J.L.M., Pinedo, H.M., 1993. Overexpression of a Mr 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Res. 53, 1475–1479.

Schinkel, A.H., 1998. Pharmacological insights from P-glycoprotein knockout mice. Int. J. Clin. Pharmacol. Ther. 36, 9–13.

Scott, K.R., Rankin, G.O., Stables, J.M., Alexander, M.S., Edafiogho, I.O., Farrar, V.A., Kolen, K.R., Moore, J., Sims, L., Tonnu, A.D., 1995. Synthesis and anticonvulsant activity of enaminones. 3. Investigation on 4′-, 3′-, and 2′-substituted amilino compounds, sodium channel binding studies and toxicity evaluation. J. Med. Chem. 38, 4033–4043.

Seelig, A., 1998. A general pattern for substrate recognition by P-glycoprotein. Eur. J. Biochem. 251, 252–261.

Sparreboom, A., van Tellingen, O., Nooijen, W., et al., 1996. Tissue distribution, metabolism and excretion of paclitaxel in mice. Anticancer Drugs 7, 78–87.

Sparreboom, A., Asperen, J.V., Mayer, U., Schinkel, A.H., Smit, J.W., Meijer, D.K.F., Borst, P., Nooijen, W.J., Beijnen, J.H., Tellingen, O.V., 1997. Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. Proc. Natl. Acad. Sci. U.S.A. 94, 2031–2035.

Sparreboom, A., van Tellingen, O., Nooijen, W., Beijnen, J.H., 1998. Preclinical pharmacokinetics of paclitaxel and docetaxel. Anticancer Drugs 9, 1–17.

Stammati, A., Zampaglioni, F., Zucco, F., 1997. Furaltadone cytotoxicity on three cell lines in the presence or absence of DMSO: comparison with furazolidone. Cell Biol. Toxicol. 13, 125–130.

Stouch, T.R., Gudmundsson, O., 2002. Progress in understanding the structure–activity relationships of P-glycoprotein. Adv. Drug Deliv. Rev. 54, 315–328.

Suzuki, H., Sugiyama, Y., 2000. Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. Eur. J. Pharm. Sci. 12, 3–12.

Valverde, M.A., Bond, T.D., Hardy, S.P., Taylor, J.C., Higgins, C.F., Altamirano, J., Alvarez-Leefmans, F.J., 1996. The multidrug resistance P-glycoprotein modulates cell regulatory volume decrease. EMBO J. 15, 4460–4468.

van der Sandt, I.C., Blom-Roosemalen, M.C., de Boer, A.G., Breimer, D.D., 2000. Specificity of doxorubicin versus rhodamine 123 in assessing P-glycoprotein functionality in the LLC-PK1, LLC-PK1:MDR1 and Caco-2 cell lines. Eur. J. Pharm. Sci. 11, 207–214.

Woo, J.S., Lee, C.H., Shim, C.K., Hwang, S.J., 2003. Enhanced oral bioavailability of paclitaxel by coadministration of the P-glycoprotein inhibitor KR30031. Pharm. Res. 20, 24–30.

Zaman, G.J., Flens, M.J., Van Leusden, M.R., de Haas, M., Mulder, H.S., Lankelma, J., Pinedo, H.M., Scheper, R.J., Baas, F., Broxterman, H.J., 1994. The human multidrug resistance-associated protein MRP is a plasma membrane drug efflux pump. Proc. Natl. Acad. Sci. U.S.A. 91, 8822–8826.

Zhou, D.C., Marie, J.P., Maisonneuve, L., Faussat-Suberville, A.M., Zittoun, R., 1993. Effect of differentiating agents on modulation of MDR1 gene expression in multidrug-resistant hematopoietic HL60/DNR cell line. Exp. Hematol. 21, 779–784.