Effect of P-Glycoprotein on the Pharmacokinetics and Tissue Distribution of Enaminone Anticonvulsants: Analysis by Population and Physiological Approaches

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Received February 21, 2002; accepted May 1, 2002

ABSTRACT
Multidrug resistance (MDR), mediated by P-glycoprotein (Pgp), has been identified as altering the disposition of structurally diverse compounds. Previous in vitro studies in bovine brain microvascular endothelial cells and MCF/Adr [Adriamycin (doxorubicin)-resistant human breast cancer] cells displayed that the transport of enaminone anticonvulsants was influenced by Pgp. Therefore the objectives of this study was to further evaluate the influence of Pgp on the pharmacokinetics and tissue distribution of the enaminone analogs, mdr1ab (+/+) and mdr1ab (−/−) male mice (20 ± 5 g) were administered DM5 (methyl 4-{[4′-chlorophenyl]amino}-6-methyl-2-oxo-3-cyclohexene-1-carboxylate) or DM44 (12.5 mg/kg, i.v.). Cohorts (n = 3) were sacrificed over a 12-h period, and samples were analyzed by a validated UV-high performance liquid chromatography assay method. Population analysis was used to estimate pharmacokinetic parameters and partition coefficients were determined for tissues. The clearance (0.51 versus 0.33 l/h/kg) and Vd (1.25 versus 0.93 l/kg) of DM5 were found to be higher (p < 0.05), however the area under the curve (26.1 versus 38.2 µg/ml·h) was lower (p < 0.05) in mdr1a/1b (−/−) versus mdr1a1b (+/+). Similar findings were observed for DM44. Tissues known to express Pgp such as the heart, liver, lung, and brain displayed 2-fold or higher tissue levels in mdr1a/1b (−/−) versus mdr1a1b (+/+). These results strongly suggest that Pgp may influence enaminone tissue distribution and pharmacokinetics and may play a significant role in the effective treatment of epilepsy with these analogs.

In addition to reducing drug accumulation in certain tissues, another effect of the MDR transporter proteins is that they can modulate the tissue distribution and pharmacokinetics of structurally diverse compounds (Schinkel et al., 1996) The influence of Pgp on pharmacokinetics and bioavailability have been evaluated by the administration of agents to genetically altered mice lacking the genes for the expression of Pgp [mdr1a, mdr1a1b (−/−)] or the coadministration of Pgp substrates with known inhibitors of the transporter protein. Pharmacokinetics studies with agents such as digoxin, cyclosporin A, dexamethasone, and vinblastine have displayed a modulation of both tissue distribution and pharmacokinetics in knockout or pretreated animals compared with wild-type mice (Schinkel et al., 1995; van Asperen et al., 1996, 1999b). Specifically, these studies have reported an increase in tissue distribution to specific tissues (i.e., brain) lacking Pgp and a decrease in total body clearance for agents such as cyclosporin A, vinblastine, and tacrolimus.

Recently, studies have examined the influence of P-glycoprotein on the distribution of various anticonvulsant agents. P-Glycoprotein has been implicated in treatment failure with

ABBREVIATIONS: Pgp, P-glycoprotein; MRP, multidrug resistance-associated protein; MDR, multidrug resistant; DMS, methyl 4-{[4′-chlorophenyl]amino}-6-methyl-2-oxo-3-cyclohexene-1-carboxylate; AUC, area under the curve; CL, clearance; CV, coefficient of variation.
epilepsy patients (Tishler et al., 1995; Lazarowski et al., 1999; Sisodya et al., 2002). Tishler et al. (1995) evaluated specimens of brains from patients undergoing surgical procedures to control intractable seizures and found that eleven of nineteen specimens had MDR1 mRNA levels 10 times greater than in normal brains (Tishler et al., 1995). These results suggest that Pgp may play a clinically significant role by exporting antiepileptic compounds from the brain. Thus, the overexpression of the MDR1 gene in the brain with these patients may contribute to their lack of response to treatment.

Enaminone anticonvulsant derivatives, as seen in Fig. 1, synthesized by Scott and investigators, represent a new and potentially active series of compounds for the treatment of generalized tonic-clonic and complex partial seizures (Scott et al., 1993, 1995). The prototype anticonvulsant of the series methyl 4-[(4'-chlorophenyl)amino]-6-methyl-2-oxo-3-cyclohexene-1-carboxylate (DM5) was found to be active intraperitoneally in mice (ED$_{50}$ 26.2 mg/kg) and orally (p.o.) in rats (ED$_{50}$ 5.8 mg/kg) and compared favorably to phenytoin under the same test conditions (ED$_{50}$ 6.5 and 23.3 mg/kg, respectively). In vitro studies evaluating the transport and/or uptake of a series of enaminones alone and in the presence of Pgp inhibitors (e.g., verapamil and rhodamine 123) in both the bovine brain microvessel endothelial cell and MCF-7/Adr cell culture systems strongly suggested that Pgp may be responsible for enaminone efflux (Eddington et al., 2000; Cox et al., 2001a). In addition, studies performed in mdr1a/b (–/–) mice lacking Pgp showed that the brain distribution of select enaminones was significantly higher ($p < 0.05$) compared with their mdr1a/b (+/+) wild-type counterparts (Cox et al., 2001b). Taken together, both the in vitro and in vivo results suggest a prominent role of Pgp in the disposition of these agents. Therefore the objectives of this study were to further evaluate 1) the influence of the MDR1 protein on the pharmacokinetics and tissue distribution (e.g., brain, heart, lungs, kidneys, and liver) of specific enaminone analogs in both wild-type and genetically altered mice; and 2) develop a population pharmacokinetic model to statistically compare parameters between groups.

**Materials and Methods**

**Materials.** Acetonitrile, methyl-butyl ether, and Na$_2$HPO$_4$ were purchased from Fisher Scientific Co. (Pittsburgh, PA). Dimethyl sulfoxide and phosphate-buffered saline were purchased from Invitrogen (Carlsbad, CA). The internal standard carbamazepine (CBZ) was purchased from Sigma-Aldrich (St. Louis, MO). Reagent alcohol (ethanol) and 1,2-propanediol were also purchased from Fisher Scientific Co. Kenneth R. Scott (Howard University, Washington, D.C.) synthesized enaminone derivatives DM5 and DM44 (Fig. 1). All other high performance liquid chromatography solvents were of analytical grade. Distilled deionized water was used in the preparation of all reagents and the mobile phase.

**Animals.** FVB wild-type mdr1a/b (+/+) and mdr1a/b (–/–) male mice weighing 20 ± 5 g (10–14 weeks of age) were utilized in all experiments (Taconic Farms, Germantown, NY). The animals were singly housed in plastic cages and were maintained in an Assessment and Accreditation of Laboratory Animal Care accredited facility operated on a 12-h light/dark cycle at a room temperature of 72 ± 2°C. Animals received Purina 5001 chow and water ad libitum except on the evening prior to dosing, when food was withheld. Care and use of the animals followed the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources (National Research Council; National Institutes of Health Publication No. 86-23). Each animal was fasted for 12 h prior to dosing.

**Pharmacokinetic Studies.** DM5 (Fig. 1A) and DM44 (Fig. 1B) were solubilized the morning of the study. Formulations for injections were as follows: DM5 and DM44 (12.5 mg/kg) were dissolved in a 2.5-ml solution of 5% reagent alcohol, 5% dimethyl sulfoxide, 22% 1,2-propanediol, and 68% phosphate-buffered saline. Final enaminone solutions were administered intravenously as a bolus dose via the tail vein at an injection volume of 0.2 ml/kg in both male mdr1a/b (+/+) and mdr1a/b knockout (–/–) mice. Cohorts of three animals were sacrificed by CO$_2$ asphyxiation at the following time points: 0.25, 0.5, 1.0, 1.5, 2.0, 4.0, 8.0, and 12.0 h. Blood samples were collected into heparinized syringes via heart puncture. Plasma was separated by centrifugation for 10 min and immediately frozen at −70°C. Tissue samples (e.g., brain, heart, lungs, liver, kidneys, and spleen) were removed, flash frozen, and stored at −70°C until analysis.

**Analytical Method.** The assay method used in the evaluation of enaminone analogs has been previously reported (Cox et al., 2000). A selective and specific high performance liquid chromatography method was developed to quantify DM5 and DM44 in plasma and tissue. Thirty microliters of the internal standard (carbamazepine, 5 µg/ml) solution was added to plasma samples/standards or to tissue homogenates samples/standards. One milliliter of ether was added to either plasma or tissue samples. The samples were vortexed briefly (1–2 min) and another 1 ml of ether was added. After the second vortexing, samples or standards were transferred to −70°C freezer for 5–10 min to freeze aqueous phase. The organic phase was then decanted into 20-ml conical test tubes. The samples were evaporated at 37°C under a gentle stream of nitrogen. The resulting residue was reconstituted in 0.5 ml of mobile phase, vortexed, and centrifuged. The supernatant was transferred to microvials (150 µl) and 30 µl of standard/sample was injected onto the high performance liquid chromatography. Reverse phase chromatography with ultraviolet (λ = 307 nm) detection was utilized to quantitate the analyst. A C$_{18}$ analytical column was used, and the mobile phase consisted of acetonitrile and 0.05 M Na$_2$HPO$_4$ buffer (60:40, v/v). The calibration curves were found to be linear ($r > 0.9999$) in the range of 0.1 to 5.0 µg/ml or µg/g. The limit of detection with a signal to noise ratio of 3:1 was 20 ng/ml. Intra-run precision was in all in the range of 5 to 10%. The absolute recovery of the analyte in tissue and plasma samples was ≥90%.

**Pharmacokinetic Analysis.** Data obtained after the administration of the enaminones (DM5 or DM44) to FVB mdr1a/b (+/+) and mdr1a/b (–/–) mice were initially analyzed by the naive pooled method. Plasma concentration versus time data from a given treatment was pooled and analyzed according to nonlinear least squares. Compartment modeling was used to estimate various pharmacokinetic parameters ($V_d$, $k_{el}$, AUC$_{\text{inf}}$, $t_{1/2}$, and CL) using WinNonLin (version 3.1; Pharsight Corp., Mountainview, CA). Both one- and two-compartment analysis were evaluated to determine the best model fit. Various weighting schemes included a weight of 1, $1/Y$ (where Y is the drug concentration), $1/Y^2$ and $1/predicted
concentration (iterative reweighting) and 1/predicted concentration squared. Goodness of fit was based on visual inspection, final residual sum of squares, weighted residual sum of squares, random distribution of residuals, Akaike information criteria, and Schwartz criteria.

Pharmacostatistical Analysis of Destructive Sample Data. To evaluate variability and statistically determine whether the pharmacokinetics of the enaminone analogs were significantly altered by Pgp, population pharmacokinetic analysis using WinNonMix version 1.0 (Pharsight Corp.) was used to determine the inter-animal variability of the pharmacokinetic parameters. (Ètte et al., 1994, 1995) The first-order estimation method was used to obtain population parameter estimates. A two-stage analysis (naive pooled data method) with the individual pharmacokinetic data was performed initially, and it was determined that a one-compartment model best described the disposition of both DM5 and DM44 after a single i.v. bolus dose. Various statistical models were assessed (e.g., additive, exponential, combined additive, and proportional). The proportional error model was used to describe the inter-animal variability in the pharmacokinetic parameters clearance (CL) and volume of distribution (V) such as (e.g., V)

\[ V_i = V \cdot (1 + \eta) \]

where \( V_i \) is the parameter of interest for the \( i \)th animal, \( V \) is the parameter estimate for the “typical” animal, and \( \eta \) is the deviation of the animal’s parameter estimate from the typical value. Several models were used to define the inter-animal variance-covariance matrix, \( \Omega \). For example, diagonal and full matrices were utilized first.

Random residual variability was modeled using a combined additive and proportional error model as follows.

\[ C_{ij} = C_{pij}(1 + \varepsilon_{ij}) + \varepsilon_{2i} \]

where \( C_{ij} \) is the \( j \)th observation for the \( i \)th animal, \( C_{pij} \) is the \( j \)th prediction concentration for \( i \)th animal, \( \varepsilon_{ij} \) is the residual intra-animal error term and is assumed to be randomly normally distributed with zero mean and variance of \( \sigma^2 \). The covariates type and weight were added one at a time in a full/reduced fashion. The more advanced model was accepted if the minimum objective function value differed by \( \pm 3.84 (p < 0.05, \chi^2 \text{ distribution}; 1 df) \). S-Plus version 4.5 (Mathsoft, Inc., Data Analysis Products Divisions, Seattle, WA) was used for goodness-of-fit diagnostics and for graphical data displays. Last, the pharmacokinetic parameters and the tissue distribution data were statistically compared between \( \text{mdr1a/1b} \) (-/-) and \( \text{mdr1a/1b} \) (+/+) using Student’s t test.

Partition Coefficients. Partition coefficients (\( R_i \)) were determined for each tissue. The coefficients were determined by the following method: ratio of the AUC\(_{0-12h}\) of each tissue versus time profile compared with the AUC\(_{0-12h}\) for the plasma versus time profile according to the following equation.

\[ R_i = \frac{\text{AUC}_{0-12h} \text{(Tissue)}}{\text{AUC}_{0-12h} \text{(Plasma)}} \]

The AUC for both plasma and tissue levels was determined by the model independent program, LAGRAN. This program utilizes the Lagrange techniques alone or in conjunction with linear or trapezoidal methods.

Results

Compartment Analysis. The observed and predicted plasma concentrations of DM5 after intravenous bolus administration (12.5 mg/kg) to \( \text{mdr1a/1b} \) (+/+) and \( \text{mdr1a/1b} \) (-/-) are depicted in Fig. 2, A and B, respectively. The goodness-of-fit criteria (e.g., \( R^2 \), Akaike information, and Schwartz) supported a one-compartment model and as seen in Fig. 2 the DM5 concentrations declined in a mono-exponential manner in both groups. A one-compartment, first-order pharmacokinetic model also appeared to fit the plasma-time profiles for DM44 in both \( \text{mdr1a/1b} \) (+/+) and \( \text{mdr1a/1b} \) (-/-) mice as seen in Fig. 2, C and D, respectively. This model was again based on goodness-of-fit criteria. Due to the destructive sampling design of the study, only trends in the pharmacokinetic parameters could be evaluated using nonlinear least-squares regression. The objective of the study was to examine which, if any, pharmacokinetic parameters might be influenced by the expression of or lack thereof of Pgp. For this reason, to determine whether Pgp played a significant role in the disposition of these two enaminones, statistical differences between parameter estimates needed to be evaluated. To achieve this goal, a population analysis was applied to these destructive sampling data. The one-compartment model from the nonlinear least-squares regression model, and parameter estimates were used for the population analysis.

Population Pharmacokinetic Analysis. The destructive sampling approach of the study allowed for the analysis of enaminone concentrations in a variety of tissues but was limiting in that it only provided one sample on the concentration-time profile for each animal. Therefore, a population pharmacokinetic analysis approach utilizing WinNonMix was used for statistical comparison of the pharmacokinetic parameters between both groups of mice (Ètte et al., 1994, 1995; Carapetis et al., 2001). Table 1 displays the chronology of the modeling procedure used to characterize the population pharmacokinetics for DM5 in both wild-type and knockout mice. A proportional error model was used to describe the inter-animal variability, and the combination additive and proportional error model were used to characterize the residual random effects. The final model also included the identification of the covariate genetic type (e.g., \( \text{mdr1a/1b} \) (+/+) or (-/-)) on clearance. Parameter estimates generated are summarized in Table 2. In general, all pharmacokinetic parameter estimates had CV% < 10 and residual standard error < 35%. The goodness-of-fit plots as depicted in Fig. 3, A and B, included predicted versus observed concentrations and weighted residual versus predicted concentrations for DM5 in both \( \text{mdr1a/1b} \) (+/+) and \( \text{mdr1a/1b} \) (-/-) mice.

The chronology of the modeling procedure used to characterize DM44 in wild-type and knockout mice is also displayed in Table 1. All pharmacokinetic parameter estimates had CV% < 16% and the residual standard error < 40%. A proportional error model was used to describe the inter-animal variability, and the combination additive and proportional error model were used to characterize the intra-animal error. The final model included the identification of the covariate genetic type (e.g., \( \text{mdr1a/1b} \) (+/+) or (-/-)) on clearance and volume of distribution. The observed versus predicted and weighted residual versus predicted goodness-of-fit plots from the final model for DM44 in both \( \text{mdr1a/1b} \) (+/+) and \( \text{mdr1a/1b} \) (-/-) mice are depicted in Fig. 3, C and D, respectively.

The primary goal in utilizing population analysis was to statistically assess the effect of Pgp on the pharmacokinetics of DM5 and DM44 in mice given the destructive sampling study design. Table 2 presents the parameter estimates using WinNonMix for both DM5 and DM44. The volume of distribution (\( V_d \)) for DM5 was significantly higher (\( p < 0.05 \)) in \( \text{mdr1a/1b} \) (-/-) (1.25 liters/kg) compared with \( \text{mdr1a/1b} \) (+/+) (1.2 liters/kg).
Likewise, correlating with the $V_d$, the AUC$_{\text{inf}}$ was significantly higher in $\text{mdr1a/1b}$ $(+/-)$ (38.2 g/ml h) than in their $\text{mdr1a/1b}$ $(--)$ counterparts (26.1 g/ml h). The pharmacokinetic parameters determined for DM44 followed the same trends as observed for DM5. The $V_d$ was significantly higher in $\text{mdr1a/1b}$ $(--)$ (14.4 liters/kg) compared with $\text{mdr1a/1b}$ $(+/-)$ (9.87 liters/kg). In addition, the AUC was significantly higher in those mice expressing Pgp (3.71 versus 2.46 g/ml h). As seen in Table 2, the DM5 and DM44 clearance were both significantly higher in the knockout animals (0.51 and 5.73 liters/h · kg, respectively) versus the wild-type mice (0.33 and 4.23 liters/h · kg).

### Tissue Distribution of Enaminone Anticonvulsants

In addition, to altering the pharmacokinetic disposition of various agents, research suggests that the expression of Pgp in various tissues in the body such as the heart, brain, lung, and liver minimizes drug distribution into these tissues (Schinkel et al., 1994; van Asperen et al., 1996; Ling, 1997). An additional objective of this study was to examine the influence of Pgp on the enaminone tissue distribution into a variety of tissues known to express Pgp. Table 3 presents the extent (AUC$_{0-12h}$) and rate ($C_{\text{max}}$, $T_{\text{max}}$) of DM5 and DM44 in both $\text{mdr1a/1b}$ $(+/-)$ and $\text{mdr1a/1b}$ $(--)$ mice after a 12.5 mg/kg i.v. dose. The rank order of DM5 tissue distribu-
Enaminone pharmacokinetics for DM5 and DM44 after a 12.5 mg/kg i.v. bolus dose in mdr1a/1b (+/+) and mdr1a/1b (−/−) determined from population analysis (WinNonMix, version 3)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DM5</th>
<th>DM5</th>
<th>DM44</th>
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<tr>
<td></td>
<td>mdr1a/1b (+/+)</td>
<td>mdr1a/1b (−/−)</td>
<td>mdr1a/1b (+/+)</td>
<td>mdr1a/1b (−/−)</td>
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<td>Vₐ (liters/kg)</td>
<td>0.93 (0.001)</td>
<td>1.25* (0.021)</td>
<td>9.87 (0.65)</td>
<td>14.4* (0.85)</td>
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<tr>
<td>Kₑ (h⁻¹)</td>
<td>0.36 (0.01)</td>
<td>0.42 (0.04)</td>
<td>0.42 (0.03)</td>
<td>0.36 (0.06)</td>
</tr>
<tr>
<td>AUC (µg/ml · h)</td>
<td>38.2* (7.41)</td>
<td>26.1 (5.63)</td>
<td>3.71* (0.06)</td>
<td>2.48 (0.12)</td>
</tr>
<tr>
<td>t₁/₂ (h)</td>
<td>1.96 (0.24)</td>
<td>1.62 (0.34)</td>
<td>1.64 (0.003)</td>
<td>1.94 (0.001)</td>
</tr>
<tr>
<td>CL (liters/h · kg)</td>
<td>0.33 (0.03)</td>
<td>0.51* (0.01)</td>
<td>4.23 (0.23)</td>
<td>5.73* (0.39)</td>
</tr>
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</table>

* Significant difference at p < 0.05.

**Fig. 3.** Goodness of fit plots for population modeling of DM5 and DM44. A, observed versus predicted DM5 plasma concentrations; B, weighted residuals versus predicted DM5 concentrations; C, observed versus predicted DM44 plasma concentrations; and D, weighted residuals versus predicted DM44 concentrations.

Enaminone pharmacokinetics for DM5 and DM44 after a 12.5 mg/kg i.v. bolus dose in mdr1a/1b (+/+) and mdr1a/1b (−/−) determined from population analysis (WinNonMix, version 3).

Table 2 shows the pharmacokinetic parameters for DM5 and DM44 in mdr1a/1b (+/+) and mdr1a/1b (−/−) mice. The table includes parameters such as volume of distribution (Vₐ), elimination rate constant (Kₑ), area under the curve (AUC), and terminal half-life (t₁/₂). The results indicate that both mdr1a/1b (+/+) and mdr1a/1b (−/−) mice have different pharmacokinetic profiles.

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Enaminone pharmacokinetics for DM5 and DM44 after a 12.5 mg/kg i.v. bolus dose in mdr1a/1b (+/+) and mdr1a/1b (−/−) determined from population analysis (WinNonMix, version 3).

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Enaminone pharmacokinetics for DM5 and DM44 after a 12.5 mg/kg i.v. bolus dose in mdr1a/1b (+/+) and mdr1a/1b (−/−) determined from population analysis (WinNonMix, version 3).

Table 2 shows the pharmacokinetic parameters for DM5 and DM44 in mdr1a/1b (+/+) and mdr1a/1b (−/−) mice. The table includes parameters such as volume of distribution (Vₐ), elimination rate constant (Kₑ), area under the curve (AUC), and terminal half-life (t₁/₂). The results indicate that both mdr1a/1b (+/+) and mdr1a/1b (−/−) mice have different pharmacokinetic profiles.

**Fig. 3.** Goodness of fit plots for population modeling of DM5 and DM44. A, observed versus predicted DM5 plasma concentrations; B, weighted residuals versus predicted DM5 concentrations; C, observed versus predicted DM44 plasma concentrations; and D, weighted residuals versus predicted DM44 concentrations.
to-plasma ratios were additionally assessed to interpret possible changes in the pharmacokinetics and distribution of enaminone analogs in both wild-type and knockout mice. DM5 tissue-to-plasma ratios for \( mdr1a/1b \) (\( /H11002/ \)) and \( mdr1a/1b \) (\( /H11001/ \)) are found in Fig. 4. As seen in Fig. 4, DM5 tissue-to-plasma concentrations were significantly higher at three time points for the heart (Fig. 4A), six points for the lung (Fig. 4B), six points for the kidney (Fig. 4C), five points for the brain (Fig. 4D), and seven points for the liver (Fig. 4E) after dosing in the \( mdr1a/1b \) (\( /H11002/ \)) mice. In addition, higher DM5 partition coefficients (Table 3) were observed for brain, lung, liver, kidney, and heart of \( mdr1a/1b \) (\( /H11002/ \)) mice.

Table 3 also depicts the tissue pharmacokinetics for DM44 in both wild-type and knockout mice. The rank order of DM44 tissue distribution was brain > spleen > lung > kidney > heart > liver. This is in contrast to our results with DM5 where the most extensive distribution was observed in the heart. The ratio of tissue distribution to Pgp-expressing tissues in the wild-type and knockout animals was 81 versus 85%, respectively. Furthermore, brain (AUC\(_{0-12h}\) = 5.4 versus 3.0 \( \mu g/g \cdot h \)), liver (0.50 versus 0.06 \( \mu g/g \cdot h \)), kidney (0.6 versus 0.24 \( \mu g/g \cdot h \)) and heart (0.58 versus 0.21 \( \mu g/g \cdot h \)) all displayed tissue concentrations at least two-fold higher in knockout mice compared with their wild-type counterparts, respectively. This was also observed in the partition coefficients for the brain, liver, and heart (Table 3). However, the

### Table 3

<table>
<thead>
<tr>
<th>DM5 Parameters</th>
<th>DM44 Parameters</th>
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<tbody>
<tr>
<td>( AUC_{0-12} )</td>
<td>( AUC_{0-12} )</td>
</tr>
<tr>
<td>( C_{max} )</td>
<td>( C_{max} )</td>
</tr>
<tr>
<td>( T_{max} )</td>
<td>( T_{max} )</td>
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<td>( R_i )</td>
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<td>----------------</td>
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</tr>
<tr>
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<td>Heart</td>
<td>276</td>
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</table>

\( ^a \) Trapezoidal method.  
\( ^b \) Raw data determination. 
WT, \( mdr1a/1b \) (\( /H11001/ \)) mice; KO, \( mdr1a/1b \) (\( /H11002/ \)) mice.

\( AUC_{0-12} \), area under the concentration-time curve from 0 to 12 hours; \( C_{max} \), maximum concentration; \( T_{max} \), time to maximum concentration; \( R_i \), partition coefficient.

**Fig. 4.** Tissue-to-plasma concentration ratios in \( mdr1a/1b \) (\( +/- \)) and \( mdr1a/1b \) (\( -/- \)) knockout mice after a single i.v. dose (12.5 mg/kg) of DM5. A, heart-to-plasma concentration ratios; B, lung-to-plasma concentration ratios; C, kidney-to-plasma concentration ratios; D, brain-to-plasma concentration ratios; E, liver-to-plasma concentration ratios; and F, spleen-to-plasma concentration ratios. Data listed as the mean at \( n = 3; \) *, significance at \( p < 0.05; ***, \) significance at \( p < 0.01. \)
spleen and lung depicted no apparent concentration differences between the knockout and wild-type mice. The extent of tissue distribution from plasma appeared to be significant for the \textit{mdr1a/1b} (\textit{+/+}) animals. As seen in Fig. 5, DM44 tissue-to-plasma concentrations were significantly higher in the kidney (Fig. 5C), brain (Fig. 5D), and liver (Fig. 5E) for a majority of the time points in the knockout animals. Taken together, these results along with the pharmacokinetic analysis discussed above suggest that Pgp effects the tissue distribution of enaminone analogs.

**Discussion**

Multidrug resistance mediated by Pgp has been identified as minimizing the blood to brain transport of numerous structurally diverse compounds. It has been reported that vincristine, doxorubicin, and cyclosporin A are each actively effluxed out of the brain by Pgp localized in the blood-brain barrier (Schinkel et al., 1996). Recently the overexpression of multidrug transporters such as Pgp has been implicated as a contributing factor in drug resistance observed with epilepsy. Studies performed in surgically resected epileptogenic human brain tissue found that the MDR1 mRNA levels were approximately 10 times greater than those in normal brain (Tisher et al., 1995). In addition, the intracellular phenytoin concentration of MDR1-overexpressing neuroectodermal cells was one-fourth of that found in MDR1-negative cells. Furthermore, studies conducted in \textit{mdr1a} (\textit{−/−}) mice have reported significant differences in not only the tissue distribution of various Pgp substrates but also their pharmacokinetic properties. These observations suggest that anticonvulsant therapy with Pgp substrates (e.g., phenytoin) may be significantly altered due to the expression of Pgp in the brain and other relevant tissues.

Enaminone anticonvulsants have been previously evaluated as compounds with potent anticonvulsant activity at the sodium channel binding site in a manner similar to class I anticonvulsants such as phenytoin, carbamazepine, and lamotrigine (Scott et al., 1993, 1995). The prototype anticonvulsant of this series DM5 (calculated log \( \text{P}_{\text{oct/water}} = 3.23 \)) was tested in this study. It has been found to be active after i.p. dosing with an ED\(_{50} \) of 26.2 mg/kg. Previous studies evaluating the permeability of enaminones suggested that their blood-brain barrier transport was influenced by Pgp (Eddington et al., 2000; Cox et al., 2001). As such, one of the objectives of this work was to examine if Pgp altered the pharmacokinetics of two enaminones, DM5 and DM44 by using \textit{mdr1a/1b} (\textit{−/−}) and \textit{mdr1a/1b} (\textit{+/+}) mice. In terms of pharmacokinetics, the volume of distribution of a Pgp substrate should theoretically be higher in animals deficient of this transporter, since the absence of the transporter allows for higher uptake in tissues known to express Pgp. After single dose (12.5 mg/kg) i.v. administration of both DM5 and DM44, the \( V_d \) was statistically higher in \textit{mdr1a/1b} (\textit{−/−}) mice (1.25 and 14.4 liters/kg) versus wild-type (0.93 and 9.87 liters/kg) animals, respectively. In addition, the AUC\(_{\text{inf}} \) was found to be significantly lower in the \textit{mdr1a/1b} (\textit{−/−}) mice for both DM5
and DM44. The smaller AUC_{int} in the Pgp-deficient animals is most likely due to the larger V_{c} observed in these animals.

The differences in AUC_{int} reported above are in contrast to other studies using Pgp-deficient mice. The majority of the pharmacokinetic studies previously performed with Pgp-deficient mice have been with the mdr1ab (−/−) mouse not the double knockout mouse (mdr1a/b (−/−)) used in this study. Slower drug elimination has been reported for the Pgp substrates, vinblastine, doxorubicin, and paclitaxel in mdr1ab (−/−) mice compared with wild-type (Sparreboom et al., 1997; van Asperen et al., 1999a,b,c). However, a study in mdr1a/b (−/−) mice with [3H]digoxin reported a similar dose-normalized biliary excretion in both Pgp-deficient and wild-type animals (Schinkel et al., 1997). We observed no significant differences in DM5 or DM44 half-life between groups; however, the clearance (Table 2) of both DM5 and DM44 was found to be faster in the Pgp-deficient mice compared with their wild-type counterparts. Previous research on the elimination of enaminone analogs suggests that the major pathway of elimination is via the liver (Scott et al., 1995). As stated previously, numerous reports with mdr1a (−/−) mice suggest a slower clearance in the genetically altered animals.

A recent study examining the metabolism of erythromycin in genetically altered mice has also reported enhanced drug elimination as observed in our experiments. The pharmacokinetics and hepatic metabolism of erythromycin a known substrate of Pgp and CYP3A were investigated in mdr1a (−/−) and (+/+) mice (Lan et al., 2000). These investigators compared CYP3A metabolism of this agent using the erythromycin breath test in Pgp-deficient and knockout animals. The rate of 14CO₂ production, an indicator of N-demethylase activity in the liver, was reported to be 1.9-fold higher in the mdr1a (−/−) mice. This result suggests that the rate of metabolism of erythromycin was higher in the Pgp knockout animals.

Pgp is constitutively expressed on the apical brush-border epithelial cells of the intestine, the bile canalicular face of hepatocytes and the brush-border epithelium of the renal proximal tubules (Schinkel et al., 1996; Ving 1996). The expression of Pgp in various tissues affects the absorption, distribution, metabolism, and excretion of Pgp substrates. Vinblastine was found to be significantly higher in brain, liver, and heart of mdr1a (−/−) mice after intravenous dosing (van Asperen et al., 1996). Higher tissue uptake was found in the brain, heart, kidney, liver, and spleen with [3H]operamide in mdr1a (−/−) versus mdr1a (+/+) mice (Schinkel et al., 1996). Investigations performed with the double knockout mice, mdr1a/1b (−/−), observed a 2- to 9-fold higher distribution of [3H]digoxin in the heart, kidney, liver, spleen, lung, and brain compared with wild-type animals. The results found in the study reported herein are comparable to the majority of tissue distribution results with mdr1a (−/−) and mdr1a/1b (−/−) mice except for DM5 which displayed lower distribution to both the spleen and heart. However, consistent with previous studies, higher distribution of DM5 was observed for the brain, lung, and liver of the mdr1a/b (−/−) mice. In contrast, there was an approximate 4-fold higher distribution of DM44 to the heart as indicated by the partition coefficient ratio of mdr1a/b (−/−)/mdr1a/1b (−/−). In addition, the majority of tissues evaluated after DM44 dosing were found to have higher partitioning in the mdr1a/ab (−/−) mice. The tissue distribution results of the liver may be supportive of the high clearance observed in the knockout animals. The liver partition coefficient ratio for DM5 and DM44 was 3.23 and 11, respectively. As noted previously in the pharmacokinetic results, the clearance of both DM5 (0.51 versus 0.33 liters/h·kg) and DM44 (5.73 versus 4.23 liters/h·kg) was significantly higher in the knockout animals. In addition, a recent study by Schuetz et al. (2000) observed a significantly higher expression of hepatic cytochromes P-450 in mdr1a/1b mice versus wild-type in a study conducted in Amsterdam. Even though these results were not duplicated in studies performed in the United States, this difference in the expression of cytochrome P-450 enzymes, may explain the significantly higher clearance of both DM5 and DM44 in the mdr1a/1b mice.

In conclusion, research evaluating the efficacy of antiepileptic agents such as phenytoin, phenobarbital, and lorazepam has suggested that MDR1 gene expression may be responsible for drug-resistant epilepsy (Tishler et al., 1996; Lazarowski et al., 1999). Previous in vitro studies of enaminone transport and uptake of enaminone analogs across the blood-brain barrier strongly suggests that the efflux protein, Pgp, influences the distribution of these agents (Cox et al., 2001a). The objectives of this study were to evaluate the influence of the MDR1 protein on the pharmacokinetics and tissue distribution of DM5 and DM44 in mdr1a/1b (−/−) mice. The clearance and V_{c} of both agents were found to be significantly higher and the AUC significantly lower in mdr1a/1b (−/−) mice. In addition, many of the tissues known to express Pgp such as the heart, liver, lung, and brain showed higher tissue levels in knockout animals compared with their wild-type counterparts. Hence, these results strongly suggest that Pgp may influence enaminone tissue distribution and pharmacokinetics and may play a significant role in the effective treatment of epilepsy with these analogs.

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