JPET Fast Forward. Published on April 29, 2003 as DOI:10.1124/jpet.103.049452

MODULATION OF P-GLYCOPROTEIN TRANSPORT ACTIVITY IN THE MOUSE BLOOD-BRAIN BARRIER BY RIFAMPIN

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JPET Fast Forward. Published on April 29, 2003 as DOI: 10.1124/jpet.103.049452
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Running title: Modulation of BBB P-glycoprotein by Rifampin

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Number of text pages: 36
Number of tables: 0
Number of figures: 8
Number of references: 32
Number of words in Abstract: 246
Number of words in Introduction: 702
Number of words in Discussion: 1032

Abbreviations: P-gp, P-glycoprotein; MDR, multidrug resistance; BBB, blood-brain barrier; Cl_{up}, uptake clearance.

Recommended section: Absorption, Distribution, Metabolism, & Excretion

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ABSTRACT

The objective of the present study was to examine the time course and concentration dependence of modulation of P-glycoprotein (P-gp) activity in the blood-brain barrier (BBB) with consequent influence on substrate uptake into brain tissue. Potential P-gp inducers (rifampin and morphine) were administered subchronically to P-gp-competent [*mdr1a(+/+)*] mice to induce P-gp expression in brain; the impact of rifampin pretreatment on brain penetration of verapamil also was evaluated with an in situ brain perfusion technique. In addition, the effect of single-dose rifampin on P-gp BBB transport activity was assessed with brain perfusion utilizing verapamil and quinidine as model P-gp substrates. Chronic exposure to rifampin or morphine induced P-gp expression in mouse brain to a modest extent. However, single-dose rifampin treatment increased the brain uptake of verapamil and quinidine in *mdr1a(+/+)* mice in a dose- and concentration-dependent manner, consistent with P-gp inhibition. Maximum inhibition of P-gp-mediated efflux of verapamil by rifampin pretreatment *in vivo* (150 mg/kg) was ~55%, while there was only ~12% inhibition of P-gp-mediated efflux of quinidine at that rifampin dose. Coperfusion of rifampin at a concentration of 500 μM abolished P-gp-mediated efflux of verapamil at the BBB. However, only ~40% inhibition of P-gp-mediated efflux of quinidine was observed with coperfusion of rifampin, even at a 2-fold higher rifampin concentration (1000 μM). The present studies demonstrate that P-gp function at the BBB can be modulated by rifampin in a dose- and concentration-dependent manner. The degree to which rifampin inhibits P-gp-mediated transport is dependent on the substrate molecule.

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INTRODUCTION

P-glycoprotein (P-gp), a member of the ATP-binding cassette (ABC) transporter superfamily, is the product of the multidrug resistance (MDR) genes (Gottesman and Pastan, 1993). In humans, only the *MDR1* gene-encoded P-gp is capable of conveying resistance to a large number of compounds, while in rodents the drug transport function is shared between *mdr1a* and *mdr1b* (Hsu *et al.,* 1989; Thiebaut *et al.,* 1987). In contrast, the *MDR2* gene encodes a phospholipid transporter, the involvement of which in drug absorption or disposition is unclear (Smit *et al.,* 1993; Ruetz and Gros, 1994).

The importance of P-gp was first recognized with the occurrence of multidrug resistance during chemotherapy (Juliano and Ling, 1976). Tumor cells are protected against various cytotoxic agents due to overexpression of P-gp; the transporter reduces intracellular concentrations of P-gp substrates such as *vinca* alkaloids, anthracyclines, and taxol (Leveille-Webster and Arias, 1995). It is becoming increasingly clear that expression of P-gp in normal tissues plays an important role in the disposition and pharmacologic activity of a broad range of compounds. P-gp is expressed constitutively in the epithelial cells lining the luminal surface of many organs associated with excretory or barrier functions, *i.e.,* the hepatic bile canalicular membrane, the renal proximal tubule, and the villus-tip enterocyte in the small intestine. In addition, P-gp is expressed in the endothelial cells that comprise the blood-brain barrier (BBB) and blood-testes barrier (Cordon-Cardo *et al.,* 1989). The expression of P-gp in these tissues associated with drug absorption, distribution to sites of biologic activity, and elimination from the body has led to the hypothesis that P-gp evolved as a protective

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mechanism against a wide range of potentially toxic substances, serving to limit distribution and facilitate elimination of substrates (Ambudkar *et al.*, 1999; Schinkel, 1997).

Since the discovery of the drug efflux activity of P-gp, numerous attempts have been made to inhibit P-gp-mediated drug efflux. Initial investigations used existing compounds, such as calcium channel blockers (*e.g.*, verapamil), immunosuppressive agents (*e.g.*, cyclosporine A), and antiarrhythmic drugs (*e.g.*, quinidine). However, because of undesirable pharmacologic effects or limited *in vivo* inhibition of transport, more specific and potent "second-generation" P-gp modulators have been developed, such as the acridone carboxamide GF120918 (Hyafil *et al.*, 1993) and a non-immunosuppressive analog of cyclosporin A, PSC833 (Lemaire *et al.*, 1996).

Several recent studies have shown that P-gp expression can be upregulated in normal tissues as well as in tumor cells. Morphine increased P-gp content approximately 2-fold in rat brain after a 5-day treatment (Aquilante *et al.*, 2000). The immunosupressant cyclosporine A has been shown to increase P-gp in both liver and intestine (Prince *et al.*, 1996). Rifampin was able to induce P-gp in both *in vivo* studies in humans (Greiner *et al.*, 1999) and *in vitro* in human colon carcinoma cells (Schuetz *et al.*, 1996). Dexamethasone rapidly increased P-gp expression more than 4.5- and 2-fold in rat liver and lung, respectively, while P-gp expression was decreased 40% in kidney (Demeule *et al.*, 1999).

Based on the broad substrate specificity and tissue distribution of P-gp, modulation of P-gp activity may result in significant alterations in the pharmacokinetics and, potentially, the

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pharmacodynamics of P-gp substrates. For example, the brain:blood distribution ratio of the opioid peptide [D-Pen^{2,5}]enkephalin (DPDPE) was increased ~4-fold, and the EC_{50} was decreased ~10-fold, in *mdr1a(-/-)* mice as compared to FVB controls (Chen and Pollack, 1998). Similarly, *mdr1a(-/-)* mice evidenced enhanced brain accumulation and antinociceptive effect of morphine as compared to their gene-competent counterparts (Zong and Pollack, 2000). Although not as well studied, induction of P-gp in normal tissue also is likely to be of clinical importance. For example, P-gp induction in response to rifampin administration was implicated in the reduced pharmacodynamic response to morphine in a study of ten healthy human volunteers (Fromm *et al.*, 1997). Similarly, induction of P-gp has been proposed as a possible mechanism of resistance to antiretroviral agents (Lee *et al.*, 1998).

Although several experiments have shown that modulation of P-gp activity can influence drug disposition and action, the dynamics of P-gp modulation have yet to be addressed, especially with respect to the impact on BBB transport *per se*. The present studies were designed to evaluate modulation of P-gp transport activity in the murine BBB utilizing an *in situ* brain perfusion model.

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MATERIALS AND METHODS

Materials

[^{3}H]-Verapamil (85 Ci/mmol), and [^{14}C]-inulin (2.21 mCi/g) were purchased from NEN Life Science Products (Boston, MA). [^{3}H]-Quinidine (20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). (±)-Verapamil hydrochloride and quinidine sulfate were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were of the highest grade available from the commercial sources.

Animals

Adult CF-1 mice [*mdr1a(-/-)* and *mdr1a(+/+)*, 30-45 g] were purchased from Charles River Laboratories (Wilmington, MA) and maintained in a breeding colony in the School of Pharmacy, The University of North Carolina at Chapel Hill. Animals were housed in a temperature- and humidity-controlled room with a 12-hr light/dark cycle, and had free access to food and water. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. All experimental procedures were conducted according to the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, D.C., 1996).

Induction of P-gp expression by morphine and rifampin in mice

Morphine [10, 20 and 30 mg/kg s.c. 3x/day on days 1, 2 and 3, respectively; saline (10 µl/g) as a control] or rifampin [200 mg/kg i.p. once daily for 4 days; DMSO (4 µl/g) as a control] was administered to CF-1 mice [*mdr1a(-/-)* and *mdr1a(+/+)*, n = 3/group, 35-45 g].

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After a 24-hr washout, animals were decapitated and brain samples were harvested. Western blot analysis was performed to assess P-gp expression in the brain.

In a separate experiment, CF-1 mice [*mdr1a(+/+)*, n = 4/group, 35-45 g] were pretreated with rifampin (200 mg/kg/day i.p.) or DMSO (4 µl/g) for 4 days. After a 24-, 48-, or 72-hr washout, mice were anesthetized and prepared for brain perfusion as described below to assess P-gp function in the BBB using brain uptake of verapamil as an index of P-gp function. Mice receiving an acute dose of rifampin (200 mg/kg i.p.) 2 hr before perfusion also were included in the P-gp functional test.

Inhibition of P-gp in the BBB by pretreating mice with a single dose of rifampin

The results of the preceding experiment indicated that any induction of BBB P-gp that might have occurred in response to rifampin pretreatment was masked by concomitant P-gp inhibition. To further evaluate the dynamics of the inhibitory process, *mdr1a(+/+)* CF-1 mice (30-45 g, n = 4/group) were pretreated with rifampin [50, 75, 100, and 150 mg/kg i.p.]. P-gp-deficient animals received a 100-mg/kg i.p. dose of rifampin to control for any non-specific effects of rifampin on processes other than P-gp-mediated efflux, and DMSO (4 µl/g) was administered as a vehicle control. At 1 hr post-dose, mice were anesthetized and prepared for brain perfusion to assess P-gp function. In this experiment, the brain uptake of both verapamil and quinidine were utilized as independent indices of P-gp activity. A blood sample was obtained at the time of perfusion to determine the circulating concentration of rifampin in order to develop relationships between transport inhibition and rifampin concentration.

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Inhibition of P-gp-mediated efflux of verapamil and quinidine by coperfusion with rifampin

CF-1 mice (30-45 g, n = 4/group) were anesthetized and prepared for brain perfusion. Verapamil and quinidine brain uptake was measured to assess P-gp function during coperfusion with differing concentrations of rifampin (50, 200, and 500 μM for verapamil; 500 and 1000 μM for quinidine).

Inhibition of P-gp-mediated efflux of quinidine by coperfusion with verapamil

CF-1 mice (30-45 g, n = 4/group) were anesthetized and prepared for brain perfusion. Quinidine brain uptake was measured to examine P-gp function during coperfusion with various concentrations of verapamil (50, 200, and 1000 μM).

Western blot analysis

Freshly isolated brain tissue was processed to obtain membrane homogenate using a procedure modified from the method of Bergwerk *et al.* (1996). Briefly, brain tissue was rinsed and homogenized with a glass dounce in 4 volumes (w/v) buffer A [1 mM $NaHCO_3$, 50 μM phenylmethylsulfonyl fluoride (PMSF)] at 4^{o}C. The homogenate was diluted to a final volume of 10.6 ml/g brain with addition of buffer B (buffer A with 1 mM EDTA). Aliquots (1.5 ml) of the homogenate then were extracted with 30 ml buffer C (0.1 M Na_2CO_3, 50 μM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin) at 4^{o}C for 15 min, followed by centrifugation at 100,000 g for 1 hr. The resulting pellet was reconstituted in buffer C. Protein content of the membrane preparations was determined by the method of Lowry *et al.*

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(1951). Plasma membrane preparations were resuspended in NuPAGE™ sample buffer and aliquots (20 µl) of sample (30 µg protein) were loaded in triplicate onto a 4-12% NuPAGE™ Bis-Tris gel. Plasma membrane preparations from P-gp overexpressing intestinal cells were used as a positive control. SDS-PAGE electrophoresis was conducted on ice for 2 hr at 160 V (constant) under reducing conditions. Following electrophoresis, the samples were transferred onto PVDF membranes for 1 hr at 25 V (constant). Nonspecific binding sites were blocked with 5% nonfat dry milk in TBS-Tween 0.05% after an overnight transfer. The membranes were incubated with P-gp antibody mdr(Ab-1) (rabbit IgG, 1:1500 dilution) (Oncogene, Cambridge, MA) in TBS-Tween for 1 hr. The membranes then were washed with TBS-Tween (3 x 10 min) and incubated with a horseradish peroxidase-linked goat anti-rabbit antibody (1:3000 dilution) in TBS-Tween for 1 hr, followed by rinsing with TBS-Tween (3 x 10 min). The membrane was exposed to Amersham ECL detection agent, and band intensity was determined by densitometric analysis.

In situ mouse brain perfusion

CF-1 mice [*mdr1a(+/+)* and *mdr1a(-/-)*, 30-45 g, n=4/group] were prepared for *in situ* brain perfusion according to the method of Dagenais *et al.* (2000). Briefly, mice were anesthetized with ketamine/xylazine (140/8 mg/kg i.p.) and the right common carotid artery was catheterized (polyethylene tubing, 0.30 mm i.d. x 0.70 mm o.d.) following ligation of the external branch. The cardiac ventricles were severed immediately before brain perfusion with Krebs-bicarbonate buffer via a syringe pump (60 sec, 2.5 ml/min, pH 7.4 with 95% O_2 and 5% CO_2, 37^{o}C) containing 1 µM ^{3}H-verapamil (0.1 µCi/ml) or 1 µM ^{3}H-quinidine (0.15 µCi/ml). ^{14}C-inulin (0.3 µCi/ml) was added as a vascular space marker. The perfusion was

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terminated by decapitation and the brain was dissected on ice. The right hemisphere (~140 mg) and perfusate (~150 mg) were collected and weighed in tared 8-ml glass scintillation vials. Brain tissue was digested with 0.7 ml Solvable (Packard, Meriden, CT) at 50^{o}C overnight. Samples were mixed with 5 ml scintillation cocktail (Ultimate Gold, Packard). Total radioactivity (^{3}H and ^{14}C) was determined simultaneously in a Packard 1600TR liquid scintillation analyzer.

Quantitation of rifampin by HPLC

All samples were stored at –20^{o}C prior to analysis. Rifampin concentrations in serum were determined by reversed-phase capillary HPLC with UV detection (Agilent 1100 series). After addition of internal standard (sulindac, 10 µl of a 1 mg/ml solution) to the serum sample (50 µl), acetonitrile (100 µl) was added to precipitate proteins (centrifugation at 14,000 rpm for 3 min). The supernatant then was evaporated to dryness under nitrogen and reconstituted in 100 µl of mobile phase [acetonitrile:5 mM ammonium acetate, pH 4.0, 38:62 (v/v)], of which 10 µl was injected onto the HPLC. Chromatographic separation was achieved on a Zorbax C_8 column (1 x 150 mm, particle size 5 µm) under isocratic conditions (50 µl/min). The absorbance of column eluent was monitored at 254 nm. Standard curve was linear between 1.6 µg/ml and 200 µg/ml when 50 µl serum was extracted.

Calculation of BBB transport parameters

Parameters related to the *in situ* brain perfusion were calculated based on the method described by Smith (1996). Brain vascular volume (V_{vasc}, ml/100 g) was estimated from

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tissue distribution of ^{14}C-inulin, which is known to diffuse very slowly across the BBB, according to the following equation:

$$V_{vasc} = \frac{X^*}{C^*}$$

where X^* and C^* represent ^{14}C -inulin in the brain (dpm/100 g) and perfusate (dpm/ml), respectively. Apparent brain distributional volumes of substrates (V_{brain}, ml/100 g) were calculated as:

$$V_{brain} = \frac{X_{brain}}{C_{perf}}$$

where X_{brain} is substrate in the brain (dpm/100 g) corrected for vascular contamination (X_{total} - [V_{vasc} • C_{perf}]) and C_{perf} is substrate concentration in perfusate (dpm/ml). Initial uptake clearance of substrates (Cl_{up}, ml/100 g/min) were calculated as:

$$Cl_{up} = \frac{X_{brain}/T}{C_{perf}}$$

where T is the perfusion time (min). The P-gp effect on brain uptake was defined as the ratio of uptake clearance in the absence versus the presence of P-gp (Dagenais *et al.*, 2001):

$$BBB\ P-gp\ Effect = \frac{Cl_{up,mdr1a(-/-)}}{Cl_{up,mdr1a(+/+)}}$$

Data Analysis

Data are presented as mean ± SD. Student's *t*- test or analysis of variance (ANOVA), where appropriate, were used to determine the statistical significance of difference between experimental groups. Statistical significance was defined as $p < 0.05$.

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The degree of inhibition of P-gp-mediated efflux of verapamil or quinidine was defined as:

$$\%\ Inhibition = \frac{Cl_{up,\ mdr1a(+/+)} - Cl_{up,\ mdr1a(+/+),DMSO}}{Cl_{up,\ mdr1a(-/-),DMSO} - Cl_{up,\ mdr1a(+/+),DMSO}} \bullet 100$$

where $Cl_{up,mdr1a(+/+)}$ is the initial uptake clearance of verapamil or quinidine in rifampin-treated *mdr1a(+/+)* mice, and $Cl_{up,mdr1a(+/+),DMSO}$ and $Cl_{up,mdr1a(-/-),DMSO}$ are the initial uptake clearance of verapamil or quinidine in DMSO-treated *mdr1a(+/+)* and *mdr1a(-/-)* mice, respectively.

The concentration-dependent inhibition of P-gp-mediated verapamil or quinidine efflux data was analyzed by nonlinear least regression (WinNonlin 3.2, Pharsight, Mountain View, CA):

$$\%\ Inhibition = \frac{I_{\max} \bullet C^{\gamma}}{IC_{50}{}^{\gamma} + C^{\gamma}}$$

where I_{max} is the maximum % inhibition of P-gp-mediated efflux by rifampin, C is the perfusate concentration of rifampin, IC_{50} is the apparent half-inhibitory constant, and γ is the sigmoidicity factor.

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RESULTS

P-gp induction in mice

Western blot analysis (Figure 1) revealed modest increases in mouse brain P-gp expression by morphine or rifampin treatment. Densitometric analysis of the bands showed that P-gp content was increased approximately 40% in morphine-treated *mdr1a(+/+)* mice (140 ± 26% vs. 100 ± 5% in vehicle-treated controls; $p > 0.05$) and approximately 50% in rifampin-treated *mdr1a(+/+)* mice (149 ± 19% vs. 100 ± 7% in vehicle-treated controls; $p < 0.05$). Since changes in P-gp content associated with morphine pretreatment did not achieve statistical significance, further experiments were conducted only with rifampin.

Functional evaluation of P-gp after multiple-dose rifampin pretreatment

The initial brain uptake clearance (Cl_{up}) of verapamil in mice after pretreatment with rifampin is shown in Figure 2. Unexpectedly, verapamil Cl_{up} was significantly higher (consistent with inhibition, rather than induction, of P-gp-mediated transport) 24 hr after treatment in rifampin-treated mice as compared to controls. The effect of rifampin was not entirely diminished until 72-hr after the last rifampin dose. Further study with an acute rifampin dose (200 mg/kg i.p.) indicated a significant inhibitory effect of rifampin on P-gp function in the BBB.

Dose-dependent inhibition of P-gp by rifampin

Single-dose rifampin treatment increased both verapamil and quinidine initial brain uptake in *mdr1a(+/+)* mice in a dose-dependent manner (Figure 3). Rifampin was much less potent in inhibiting of P-gp-mediated quinidine efflux as compared to verapamil efflux.

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Maximum inhibition of P-gp-mediated efflux of verapamil by rifampin was approximately 55% (with 100% inhibition defined as uptake in *mdr1a(-/-)* mice), while there was only about 12% inhibition of P-gp-mediated efflux of quinidine at the highest rifampin dose tested. A 100-mg/kg dose of rifampin produced no effect on brain uptake of verapamil or quinidine in *mdr1a(-/-)* mice as compared to the DMSO-treated controls ($P > 0.05$). As was the case for the dose-response profile, the relationship between % inhibition of quinidine efflux and rifampin blood concentration also was shifted rightward as compared to that of verapamil efflux (Figure 4).

Influence of rifampin coperfusion on brain uptake of verapamil and quinidine

Coperfusion of rifampin resulted in a concentration-dependent increase in verapamil uptake clearance in *mdr1a(+/+)* mice (Figure 5). The apparent IC_{50} for inhibition by rifampin was about 220 μM. Rifampin at a concentration of 500 μM was able to inhibit almost completely P-gp-mediated efflux of verapamil in the BBB (verapamil Cl_{up} of 120 ± 41 ml/100 g/min in rifampin-treated *mdr1a(+/+)* mice vs. 136 ± 6 ml/100 g/min in DMSO-treated *mdr1a(-/-)* mice, $P > 0.05$).

Similar to the results obtained from the single-dose rifampin treatment experiment, coperfusion of rifampin was much less potent in inhibiting of P-gp-mediated quinidine efflux as compared to verapamil efflux. The degree of inhibition of P-gp-mediated efflux of quinidine by coperfusion of rifampin at concentrations of 500 and 1000 μM was approximately 30% and 40%, respectively (Figure 5).

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Influence of verapamil coperfusion on brain uptake of quinidine

Coperfusion of verapamil was able to inhibit P-gp-mediated quinidine efflux in the BBB (Figure 6). However, the degree of inhibition was not significantly different among different verapamil concentrations ($P > 0.05$). The pooled maximum inhibition of P-gp-mediated quinidine efflux by coperfusion of verapamil was ~63%.

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DISCUSSION

Due to the central role of P-gp in the absorption, distribution, and elimination of xenobiotics, an understanding of the dynamics of P-gp modulation is of importance from both a clinical and toxicological point of view. The present study was designed to investigate the influence of modulation of P-gp activity on brain transport of compounds.

Rifampin was selected to induce P-gp in mouse brain because it is a potent inducer of P-gp both in human duodenal biopsies (~3.5-fold) (Greiner *et al.*, 1999) and in human colon carcinoma cell lines (Schuetz *et al.*, 1996). Morphine has been shown to increase P-gp content about 2-fold in rat brain after a 5-day treatment (Aquilante *et al.*, 2000). However, in the present studies both agents induced P-gp expression in mouse brain to only a modest extent (~40% and ~50% increase with morphine and rifampin treatment, respectively; the changes associated with morphine treatment did not achieve statistical significance). This discrepancy may due to species differences and/or tissue specificities in xenobiotic-mediated induction of P-gp expression in the brain. Such differences are not unusual. For instance, rifampin is an efficacious inducer of cytochrome P450 3A (CYP3A) in humans but not in rodents (LeCluyse, 2001). Similarly, dexamethasone increased P-gp expression more than 4.5- and 2-fold in rat liver and lung, respectively, while it decreased P-gp expression 40% in kidney (Demeule *et al.*, 1999). Further studies are required to identify more effective inducers of brain P-gp in order to study the dynamics of P-gp induction in the BBB.

The results of functional tests after P-gp induction by rifampin were unanticipated. There was a significant increase in verapamil brain uptake, rather than the anticipated

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decrease in verapamil Cl_{up}, following pretreatment with rifampin (Figure 2). This observation suggests that inhibition of P-gp by rifampin masks whatever increase in P-gp-mediated transport might occur secondary to rifampin pretreatment. It is possible that rifampin binds tightly to the brain capillaries due to its high lipophilicity, thus evidencing a prolonged residence time in the BBB or the brain parenchyma. Several early studies have shown that rifampin can inhibit P-gp activity *in vitro* (Fardel *et al.,* 1995; Furusawa *et al.,* 1997). Functional tests with an acute dose of rifampin indeed demonstrated a significant inhibitory effect on BBB P-gp (Figure 2). Therefore, induced P-gp activity by multiple doses of rifampin might actually be masked by an inhibitory effect of rifampin itself.

Further assessment of the dynamics of P-gp inhibition by acute rifampin was conducted using verapamil and quinidine as model compounds because of their moderate to high P-gp effect *in situ* (Dagenais *et al.,* 2001). These experiments revealed a dose-dependent inhibition of P-gp-mediated efflux, although the degree of rifampin-associated inhibition differed between the two substrates (Figure 3). The maximum inhibition of P-gp-mediated verapamil efflux was ~55% at a rifampin dose of 150 mg/kg, while there was only a maximum inhibition of ~12% for P-gp-mediated quinidine efflux. These results are consistent with the fact that quinidine has a higher P-gp effect than verapamil at the murine BBB. The brain uptake of both verapamil and quinidine was unaffected by a 100 mg/kg i.p. dose of rifampin in *mdr1a(-/-)* mice compared to the controls, suggesting that transporters other than *mdr1a* isoform encoded P-gp were not involved.

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Pretreatment with rifampin was only able to partially abolish P-gp activity in the BBB *in situ.* This incomplete inhibition may be due to an inability to achieve sufficiently high rifampin concentrations in the BBB after systemic administration because of dose-limiting toxicity. Alternatively, rifampin may not be able to block completely P-gp activity in the BBB. The latter hypothesis was not supported because rifampin was able to inhibit almost completely P-gp activity in the BBB during coperfusion with verapamil (Figure 5). Verapamil Cl_{up} in *mdr1a(+/+)* mice coperfused with 500 μM rifampin was not statistically different from that in *mdr1a*-gene-deficient mice ($P > 0.05$). More importantly, the degree of P-gp inhibition by pretreatment with rifampin was in good agreement with the results from the coperfusion study. When the data are plotted on the same set of axes (Figure 7), the concentration-dependent change in verapamil efflux transport is nearly identical between the two experiments. suggesting that the method of treatment was not important (*i.e.*, derived metabolites do not contribute to the inhibitory effect) and that the concentration of the inhibitor dictates the degree of inhibition. In addition, total concentrations of rifampin in the cerebral vasculature (i.e., unbound rifampin in the coperfusion study, in which the perfusate was protein-free; bound plus unbound rifampin after *in vivo* pretreatment) appear to serve as the driving force for P-gp inhibition. In contrast, rifampin appeared to be unable to inhibit completely P-gp-mediated quinidine efflux even at very high concentrations (*i.e.*, 1000 μM in the perfusate, Figure 5). The maximum inhibition based on the data from both pretreatment and coperfusion of rifampin studies was ~42% (Figure 8), which was comparable to the ability of verapamil to inhibit quinidine transport (~63% inhibition, Figure 6). These results were intriguing. As evidenced in the present study, rifampin was able to completely inhibit P-gp-mediated verapamil efflux in the BBB. In addition, quinidine has

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been shown to be able to restore verapamil brain uptake in *mdr1a(+/+)* mice to the level of *mdr1a(-/-)* mice (personal communication from Dagenais C.). However, both rifampin and verapamil were only able to partially block P-gp-mediated quinidine efflux in the BBB. It has been suggested that there are multiple binding sites on the P-gp (Martin *et al.*, 2000; Shapiro *et al.*, 1999). One possible explanation for the current observation is that verapamil and rifampin may bind to the same site(s) on P-gp, while quinidine may interact with other site(s) in addition to the verapamil/rifampin site. This hypothesis was further supported by observations reported by Wang *et al.* (2000). They have shown that the P-gp substrate H33342 interact with quinidine in a non-competitive manner, while it interacts with verapamil in a mixed mode of inhibition.

In conclusion, the present studies have shown that P-gp function in the BBB can be modulated by rifampin in a dose- and concentration-dependent manner. The results also suggest that perturbations in the disposition of P-gp substrates in the brain may bepredicted based on the extent of P-gp modulation (*e.g.*, inhibition or induction).

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FOOTNOTES:

This project was supported in part by NIH grant *GM61191*.

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FIGURE LEGENDS

Figure 1. Representative immunodetection of P-gp in mouse brain after morphine (A) or rifampin (B) treatment. Lane1: positive control from P-gp overexpressing intestinal cell homogenate, lane 2-4: vehicle-treated *mdr1a(+/+)* mice, lane 5-7: morphine-treated (A) or rifampin-treated (B) *mdr1a(+/+)* mice, lane 8-10: morphine-treated (A) or rifampin-treated (B) *mdr1a(-/-)* mice.

Figure 2. Effect of acute rifampin administration or washout time after multiple doses of rifampin on brain uptake (mean ± SD, n = 4 per group) of verapamil (1 μM) in *mdr1a(+/+)* mice (*black bars*). No clear temporal trend was observed with time post-treatment in DMSO-treated controls. These groups were combined (n = 13) and the results, presented as a range representing the mean ± SD are shown as the horizontal gray bar. *$p < 0.05$ vs. the 72-hr washout group; **$p < 0.01$ vs. DMSO-treated controls.

Figure 3. Effect of a single dose of rifampin (50, 75, 100, or 150 mg/kg, i.p.) on brain uptake of (A) verapamil (1 μM) or (B) quinidine in *mdr1a(+/+)* mice (black bar) or *mdr1a(-/-)* mice (gray bar). Data are presented as mean ± SD (n = 4 per point). *$p < 0.05$ vs. DMSO-treated *mdr1a(+/+)* controls. There was no statistical difference between DMSO-treated and rifampin-treated (100 mg/kg, i.p.) *mdr1a(-/-)* mice ($p > 0.05$).

Figure 4. Dose-dependence (A) and concentration-dependence (B) of rifampin-associated inhibition of P-gp-mediated efflux of verapamil (circles) or quinidine (triangles). Maximum inhibition of P-gp-mediated efflux of verapamil and quinidine by rifampin was approximately 55% and 12%, respectively. Data are presented as mean ± SD (n = 4 per point).

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Figure 5. Effect of coperfusion with rifampin (RIF, 50, 200, 500, and 1000 μM) on brain uptake of (A) verapamil (1 μM) and (B) quinidine in *mdr1a(+/+)* mice. Data are presented as mean ± SD (n = 4 per point). *p < 0.05 vs. DMSO-treated *mdr1a(+/+)* controls. There was no statistical difference between rifampin-treated (500 μM) *mdr1a(-/-)* mice and DMSO-treated *mdr1a(-/-)* mice (p > 0.05).

Figure 6. Effect of coperfusion with various concentrations of verapamil (VER, 0, 50, 200, 1000 μM) on brain uptake of quinidine (1 μM) in *mdr1a(+/+)* mice. Data are presented as mean ± SD (n = 4 per point). *p < 0.05 vs. *mdr1a(-/-)* controls.

Figure 7. Concentration-dependent inhibition of P-gp-mediated verapamil efflux by rifampin. Line indicates the fit of a simple inhibitory model ($\gamma = 1$) to the coperfusion data (open triangles) shown in Figure 5; closed circles indicate data from rifampin pretreatment experiment (Figure 4). Kinetic parameters associated with the model are: I_{max} (120.3 ± 21.7 %); IC_{50} (220.0 ± 94.9 μM).

Figure 8. Concentration-dependent inhibition of P-gp-mediated quinidine efflux by rifampin. Line indicates the fit of a sigmoidal inhibitory model to the coperfusion data (open triangles) shown in Figure 5 and data from rifampin pretreatment experiment (closed circles) shown in Figure 4. Kinetic parameters associated with the model are: I_{max} (42.2 ± 8.6 %); IC_{50} (310.7 ± 119.9 μM); and γ (1.8 ± 0.6).

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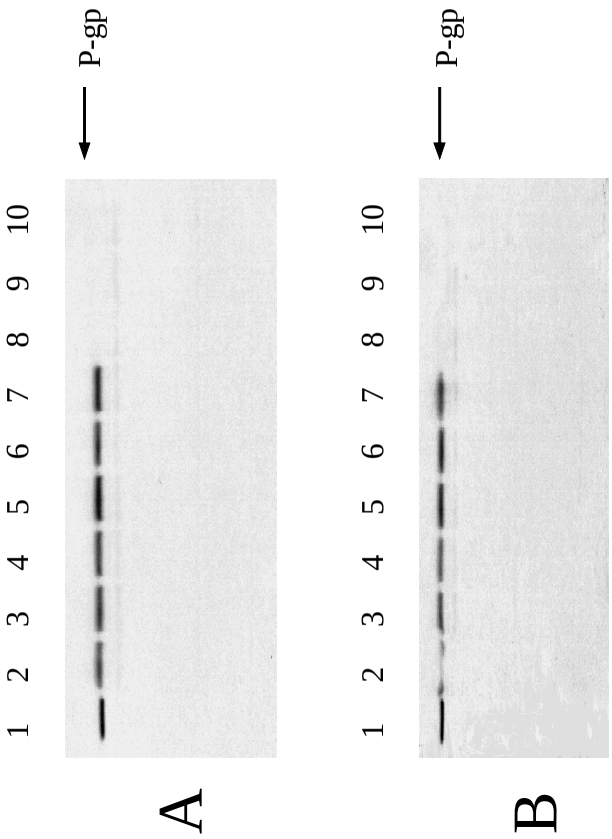

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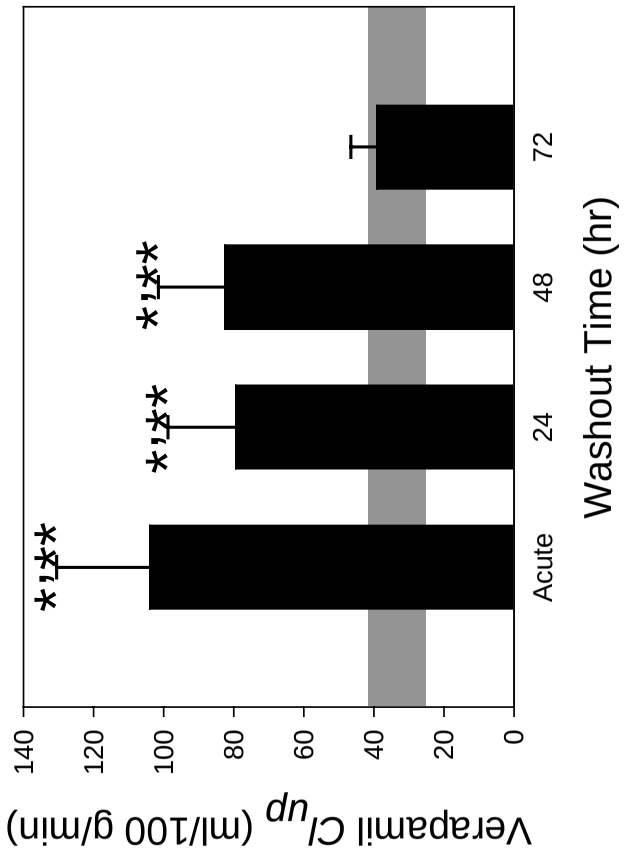

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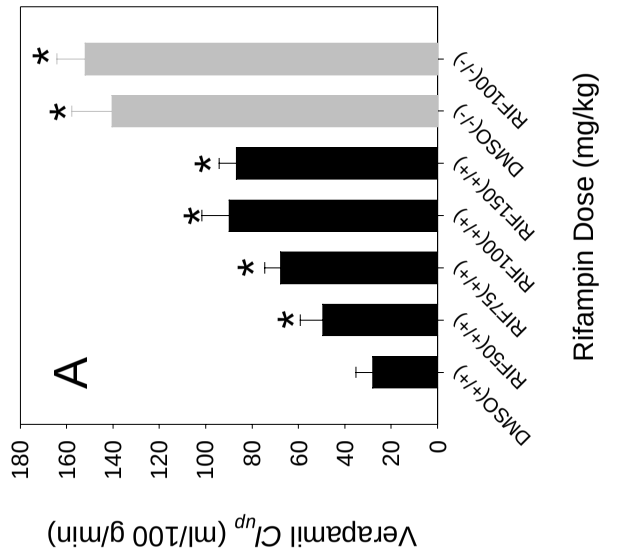

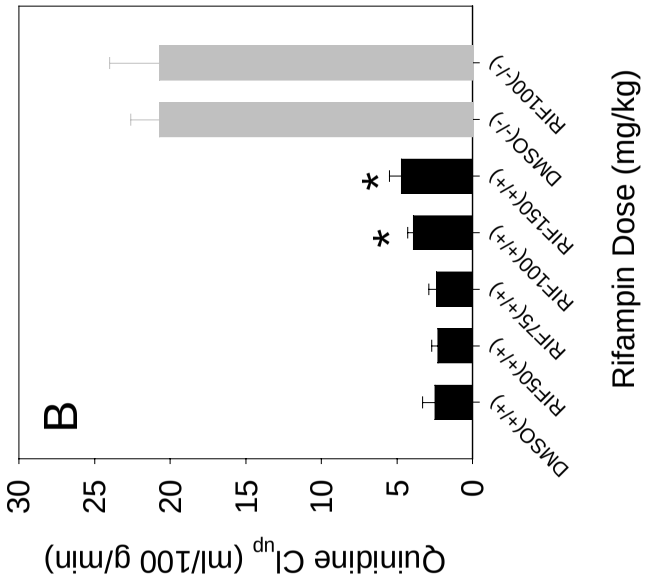

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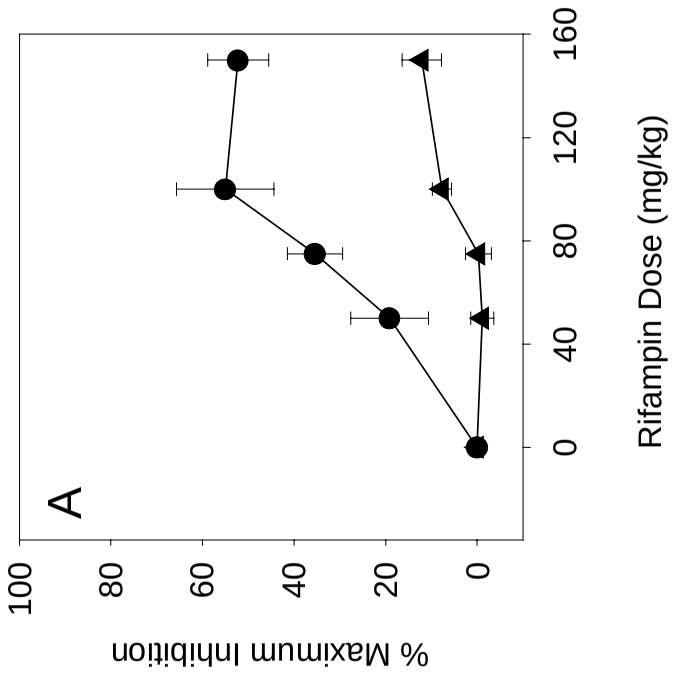

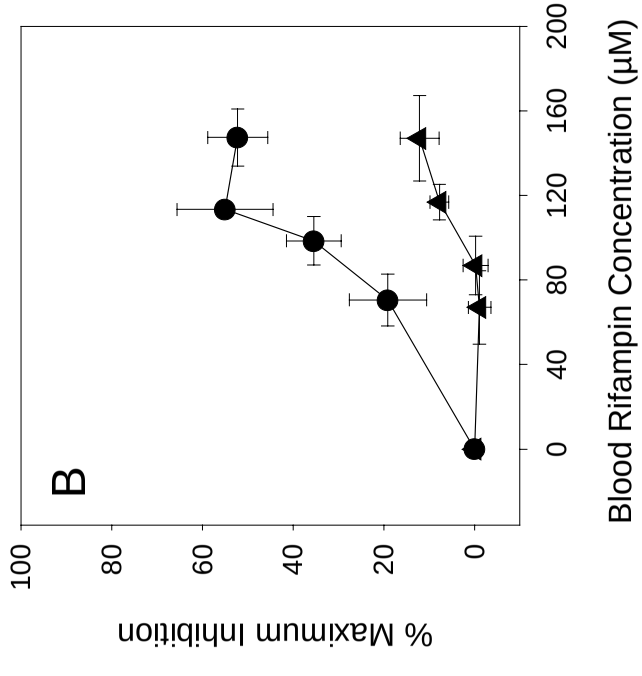

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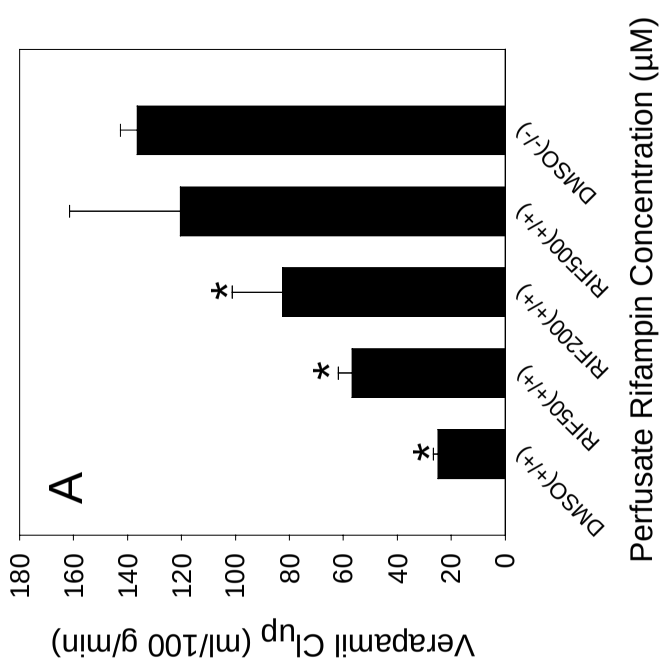

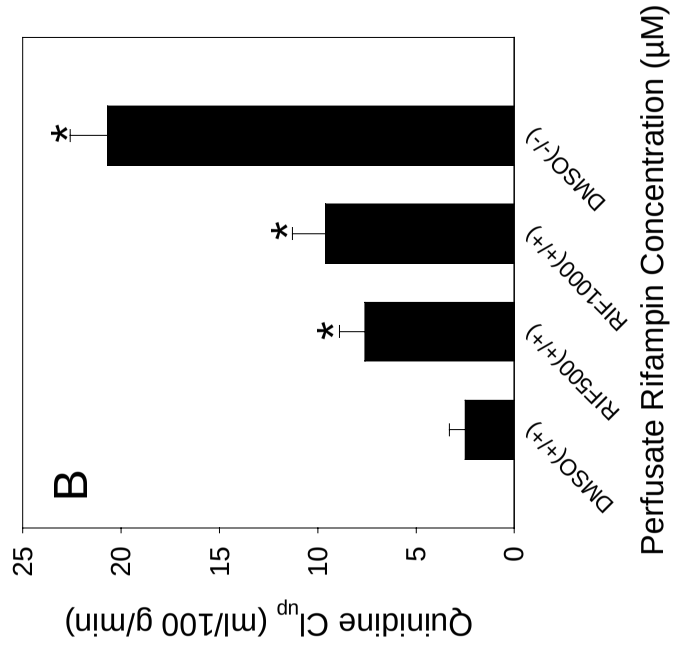

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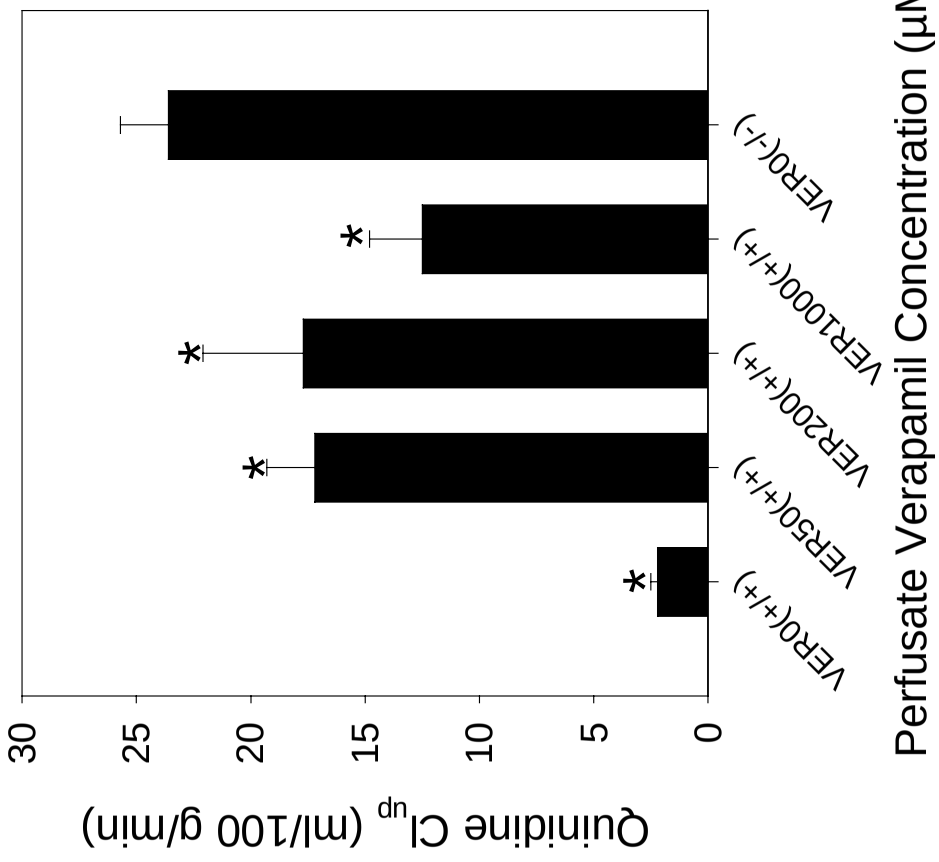

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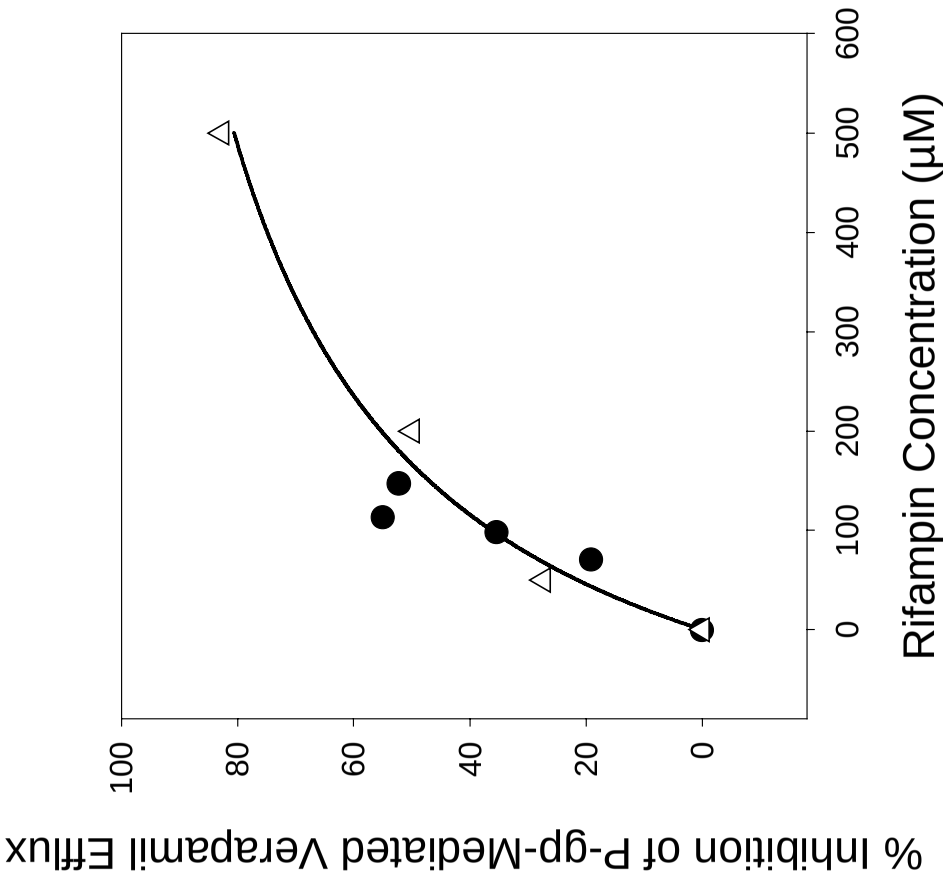

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