P-Glycoprotein Attenuates Brain Uptake of Substrates after Nasal Instillation

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Purpose. Previous literature has suggested the absence of an effective barrier between the nasal mucosa and the brain for compounds administered via the nasal route. These experiments were conducted to elucidate the role of the blood–brain barrier efflux transporter P-glycoprotein (P-gp) in attenuating delivery of P-gp substrates to the brain after nasal administration in mice.

Methods. Brain uptake of several radiolabeled P-gp substrates, was measured in P-gp-deficient and P-gp-competent mice following nasal instillation. Additional experiments were performed to assess the potential for enhancing brain uptake by inhibiting P-gp with intranasal rifampin.

Results. All substrates examined were measurable in brain tissue within 2 min. Substrate accumulation in P-gp-deficient mice was higher than in P-gp-competent animals; the degree to which P-gp attenuated brain uptake after nasal administration was similar to that during *in situ* brain perfusion. Co-administration of rifampin enhanced brain uptake of relevant substrates, and resulted in complete elimination of P-gp-mediated transport for ^{3}H- verapamil.

Conclusions. P-gp attenuates brain accumulation of intranasally-administered P-gp substrates. Thus, biochemical components of the blood–brain barrier, such as efflux transporters may influence brain penetration after nasal administration. Co-administration of a P-gp inhibitor enhances the brain uptake of relevant substrates, suggesting that the transporter barrier functions may be reversible.

KEY WORDS: P-glycoprotein; nasal administration; blood–brain barrier.

INTRODUCTION

Delivery of drugs to the central nervous system remains a challenge despite a significant improvement in our understanding of the mechanisms governing the maintenance of brain homeostasis. Specifically, the blood–brain barrier (BBB) constitutes the major interface between blood and brain and functions in part to limit xenobiotic penetration into the parenchymal compartment. The BBB is a primary determinant of substrate permeation into the brain due to several factors, including substrate lipophilicity, molecular size, ionic charge, and specificity for a variety of ATP-dependent transport systems in the influx and/or efflux direction (1). In general, the BBB limits penetration either by increasing efflux or attenuating influx.

Nasal administration of substrates has long been explored as a means to deliver agents directly to the central nervous system (CNS). A drug administered by the nasal route may enter primarily into the blood of the general circulation, primarily into the brain, or in some cases into both to varying extents (2). However, the therapeutic potential of this mode of administration has not been fully realized. This may be due, in part, to the fact that the prevailing hypothesis, i.e., that nasal administration allows direct delivery to the CNS by bypassing the BBB, is not completely accurate in its premise. Although some drugs may be delivered directly to the brain parenchymal tissue via the nasal route, the BBB, including relevant efflux transport systems, may be operative at this site. Many of the factors controlling substrate flux through each of these pathways remain unclear. In general, there are three routes that a drug administered into the nasal cavity may traverse, including entry into the systemic circulation directly from the nasal mucosa [e.g., testosterone (3)], entry into the olfactory bulb via axonal transport along neurons (e.g., dopamine; 4), and direct entry into the brain (e.g., cocaine; 5). A substrate that enters primarily into the systemic circulation must be absorbed through the nasal mucosa. The fraction of the administered dose absorbed by this route will depend on the time of mucosal contact and on the solubility of the drug in the mucus, as well as on the rate of nasal mucus clearance (6). Administration via this route avoids hepatic/gastrointestinal first-pass effects and therefore may result in extensive absorption for substrates that have poor oral bioavailability (7). However, this particular route does not provide any additional advantage for the delivery of agents to the CNS, as the substrate must traverse the BBB from the systemic circulation.

A substrate may enter the olfactory bulb via intracellular axonal transport along the olfactory neuron. This olfactory nerve pathway allows substrates to be taken up into the neuronal cell (located in the olfactory epithelium) by endocytosis with subsequent transport into the CNS. This route is used by some metals (8), viruses (9) and proteins (10), and represents the only path by which the BBB may be bypassed entirely. Despite the ability of this route to deliver agents to the olfactory bulb, transport to CNS sites beyond the olfactory system remains unclear. Furthermore, this route is slow (approx. 2.5 mm/h in monkeys; 11), and therefore does not account for the rapid appearance of some solutes in the brain/CSF following nasal administration.

Finally, the mechanisms governing the apparent direct delivery of substrates to the CNS have yet to be elucidated. This route purportedly involves substrate delivery directly to the brain parenchymal tissue, to the cerebrospinal fluid (CSF), or to both (2) after entry into the olfactory epithelium at some point other than the affector neuron (12). Although compounds may be able to enter the perineural space directly from the olfactory epithelium with subsequent diffusion to the CSF, this is not likely to be a pharmacologically viable route. Diffusion of substrate through CSF into brain tissue would be countercurrent to flow (5), the diffusion path is long considering the rapid turnover of CSF, which completely turns over approximately every 60 min in mice and every 4 h in humans (13), and the surface area of the choroid plexus is small, approximately 1000-fold less than the BBB (14). This pathway may constitute one route of entry into brain tissue, although it cannot be the only (and is likely not the predominant) direct route. Many drugs have been detected in CSF after nasal administration (e.g., cephalexin; Ref. 15 and zi-

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dovudine; 16), but the actual pathway of entry has not been traced, and the pharmacologic implications are unknown. A comprehensive understanding of the mechanisms governing this pathway is necessary in order to investigate the potential use of nasal administration as a practical means of delivering agents to the brain. The experiments reported herein represent an initial attempt to evaluate the impact of one BBB component, P-gp-mediated efflux, on brain uptake after nasal instillation.

METHODS

Materials

Probe substrates were obtained from the following sources: [^{3}H]-(±)-verapamil (85 Ci/mmol), [^{14}C]-doxorubicin (55 mCi/mmol), [^{14}C]-diazepam (56 mCi/mmol; NEN Life Science Products, Boston, MA, USA); [^{3}H]-morphine (80 Ci/mmol), [^{3}H]-quinidine (20 Ci/mmol), [^{3}H]-naltrindole (60 Ci/mmol), [^{3}H]-meperidine (80 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO, USA); [^{3}H]-meperidine (3 Ci/mmol), [^{3}H]-ritonavir (1 Ci/mmol; Moravek Biochemicals, Brea, CA, USA); and [Tyr^{2,6}-^{3}H]-DPDPE (45 Ci/mmol) was obtained from Dupont New England Nuclear (Boston, MA, USA). All other reagents used in this study were of the highest grade available from commercial sources.

Animals

Adult CF-1 mice [*mdr1a(+/+)* and *mdr1a(–/–)*, 30–40 g, 8–12 weeks of age] were purchased form Charles River Laboratories (Wilmington, MA, USA) and maintained in a breeding colony in the School of Pharmacy, The University of North Carolina. Male and female mice were housed separately (maximum of four animals per cage) in wire-mesh cages in a temperature- and humidity-controlled room with a 12-h-dark/12-h-light cycle, and had unrestricted access to food and water. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of North Carolina, and all procedures were conducted according to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985).

Nasal Administration

Adult male CF-1 mice [*mdr1a(+/+)* and *mdr1a(–/–)*] were anesthetized by intraperitoneal injection of ketamine and xylazine (140 and 8 mg/kg, respectively). The mice were placed in a supine position with the head held back and a dowel (approx. 7 mm) placed under the neck to limit liquid flow down the trachea. Solutions containing test compounds were gassed with 95% O_2 and 5% CO_2 for pH control (7.4) and warmed to 37°C in a water bath before instillation. When appropriate, tracers were added at a concentration of 0.2 μCi/mL. The solutions were administered via separate 10-μL gastight syringes (2-inch, 23-gauge needle) to each nostril. Timing of the experiment was initiated after completion of instillation. The experiment was terminated by decapitation at predetermined time points. Following decapitation, the entire brain was removed from the skull, surface blood was blotted off and the brain was placed in a tared vial and weighed.

Time Course of Total Brain Uptake

A preliminary experiment was conducted to determine how rapidly test substrates might enter the CNS after nasal administration. Aliquots (50% methanol in saline; 10 μL per nostril) containing ^{3}H-verapamil and ^{14}C-diazepam (5 μM; 2 μCi per radionuclide) were instilled into each nostril of anesthetized P-gp-competent and P-gp-deficient mice as described above. Verapamil is an established P-gp substrate (17) while diazepam uptake into brain from the systemic circulation is limited by cerebral blood flow (18). In the current experiment, it was assumed that diazepam would be absorbed rapidly across the nasal mucosa, and therefore would serve as a useful internal standard. Control animals received vehicle only. The experiment was terminated at pre-determined time points via decapitation, the brain was excised, and total radioactivity was determined by liquid scintillation spectroscopy.

Brain Uptake of Model P-gp Substrates

A variety of established P-gp substrates was used to determine the influence of P-gp on the total brain uptake of these substrates. Substrates used included meperidine, morphine, doxorubicin, naltrindole, ritonavir, verapamil, quinidine, and DPDPE. For this experiment, each substrate was dissolved in saline to produce a final concentration of 5 μM; the dosing solution was instilled nasally at a volume of 2.5 μL/nostril. At 5 min, the animals were decapitated, the brain was excised, and total radioactivity was determined by liquid scintillation spectroscopy.

Chemical Inhibition of P-gp-Mediated Attenuation of Brain Uptake

Rifampin was selected as an acute P-gp inhibitor and was administered nasally to anesthetized mice. An initial experiment was performed to determine whether rifampin could reverse the P-gp-mediated exclusion of ^{3}H-verapamil from the CNS. P-gp-competent mice received a single dose of rifampin (500 μM in methanol; 5 μL per nostril) 10 min before administration of ^{3}H-verapamil (5 μM; 2 μCi; 2.5 μL per nostril). Animals were sacrificed 3 min after administration of ^{3}H-verapamil. Control animals received methanol (5 μL per nostril). In preliminary studies, brain uptake of ^{3}H-verapamil was similar in the presence (41.5 ± 4.5 DPM/g) vs. the absence (37.4 ± 2.5 DPM/g) of methanol. Likewise, methanol did not affect the brain uptake of quinidine (56.4 ± 7.1 vs. 51.0 ± 1.1 DPM/g in the absence vs. the presence of methanol, respectively). An additional control group of P-gp-deficient animals was examined to assess the magnitude of rifampin-associated transport inhibition in *mdr1a(+/+)* mice (uptake in the *mdr1a(–/–)* animals was assumed to be independent of P-gp-mediated flux). Because 500 μM rifampin could inhibit completely P-gp associated exclusion of ^{3}H-verapamil from the CNS after nasal instillation, a complete dose-ranging inhibition experiment was performed. Before the dose-ranging study, a time-course experiment was performed to determine the optimal time for administration of the rifampin dose relative to ^{3}H-verapamil. Rifampin (500 μM in methanol; 5 μL per nostril) was administered at varying lengths of time (2, 5, 7, 10, 15, and 20 min) prior to ^{3}H-verapamil administration (5 μM; 2 μCi; 2.5 μL per nostril) to characterize the time course

of inhibition associated with rifampin. This experiment indicated that 7 min was the minimum amount of time required for onset of significant inhibition. For the dose-ranging study, rifampin (5 μL; 1–1000 μM) was administered to each nostril of P-gp-competent mice as described in the preceding experiment. Control animals received only methanol vehicle, and P-gp-deficient animals were included to define complete inhibition of P-gp-mediated transport. After 7 min, 2.5-μL aliquots of saline containing ^{3}H-verapamil (5 μM; 2 μCi) were administered to each nostril, and the experiment was terminated 3 min later via decapitation. The brain was excised and total radioactivity was determined as described in the methods. The degree of transport inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \frac{DPM_{\text{rifampin}} - DPM_{\text{MeOH}}}{DPM_{(-/-)} - DPM_{\text{MeOH}}}$$

To determine the potential generality of these results, a parallel experiment was performed with identical design, using ^{3}H-quinidine as a probe of P-gp-mediated transport.

Determination of Total Radioactivity

After excision, the brain was blotted dry and placed in a tared scintillation vial and weighed. Tissue samples were digested in 1 mL Solvable® (Packard, Meriden, CT, USA) at 50°C overnight. After cooling, 15 mL of Ultima Gold XR® (Packard) scintillation cocktail was added, and the sample was vortex-mixed. Scintillation counting (referenced to appropriate quench curves for single- or dual-label counting, depending on the experiment) was performed in a Packard Tri-Carb model 1900 TR (Packard). Brain samples obtained from naïve mice were analyzed, and these blank samples were subtracted from all analyzed samples to correct for apparent background.

Data Analysis

Data are presented as mean ± SD for three to five mice per experimental group. Where appropriate, a two-tailed Student's *t* test was used to evaluate the statistical significance of differences between experimental groups. In all cases, $p < 0.05$ was used as the criterion of statistical significance. In relevant experiments, pharmacodynamic models (simple or sigmoidal E_{max} functions) were fit to the data by nonlinear least-squares regression (WinNonlin 3.2, Pharsight, Cary, NC, USA) to obtain estimates of key parameters (E_{max}, ED_{50}, γ). Appropriate statistical and model selection criteria were used to evaluate model performance.

RESULTS

Time Course of Brain Uptake after Nasal Instillation and Potential Role for P-Glycoprotein

In both mouse strains, ^{3}H-verapamil uptake into brain was rapid. Maximum brain content was observed within 5 min. As anticipated, uptake of ^{3}H-verapamil was slower than that of ^{14}C-diazepam, with a time-dependent increase in the ^{3}H-verapamil to ^{14}C-diazepam ratio over the first 5 min. At the 3-min time point, brain uptake of ^{3}H-verapamil was approximately 2-fold higher in the P-gp-deficient mice as compared with control animals (Fig. 1A). At each time point,

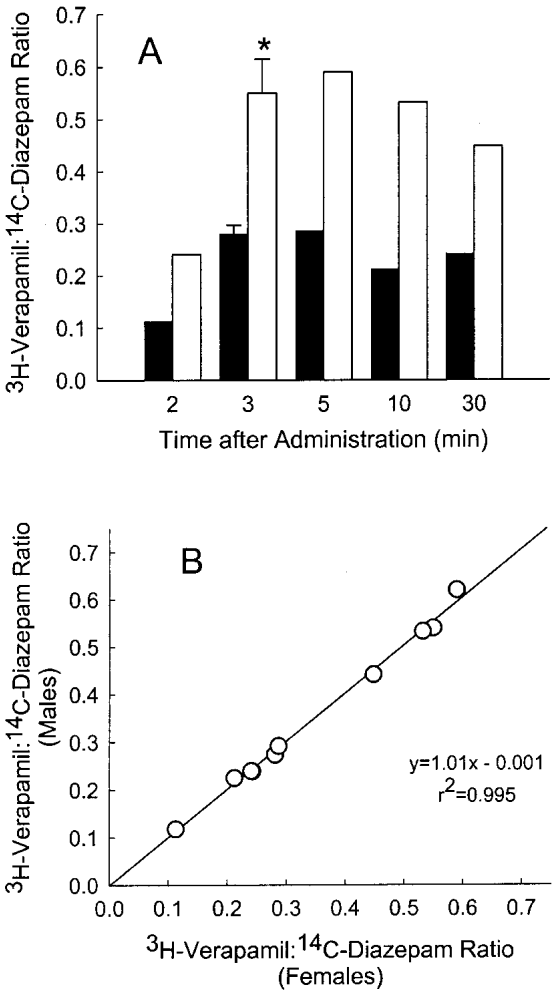

Fig. 1. Brain uptake of ^{3}H-verapamil relative to ^{14}C-diazepam (A) in *mdr1a(+/+)* (closed bars) and *mdr1a(–/–)* (open bars) mice. B, correlation between male and female mice. Data at 3 min represent mean ± SD (n = 5), *$p < 0.001$ vs. *mdr1a(+/+)*. All other time points represent the average of two observations.

there was a trend toward higher brain uptake in the *mdr1a(–/–)* mice, although this pilot experiment was not designed to draw statistical comparisons between the two mouse strains at any time point other than 3 min. In addition, there was no noticeable difference between the male and female mice (Fig. 1B), consistent with previous observations in this laboratory that indicated no gender differentiation of the P-gp effect for brain uptake during *in situ* brain perfusion in mice (19). These observations supported the hypothesis that P-gp limits the ability of P-gp substrates to access the brain via nasal administration.

Influence of Efflux Transport on Brain Uptake of Nasally-Administered P-gp Substrates

This experiment was performed to evaluate the role of P-gp in determining brain uptake after nasal administration of various P-gp substrates. For each of the substrates studied, brain uptake was increased in *mdr1a(–/–)* mice as compared to *mdr1a(+/+)* controls (Fig. 2). These experiments were performed in a manner similar to the preceding studies, with 2.5 μL of a 5-μM solution administered per nostril with uptake

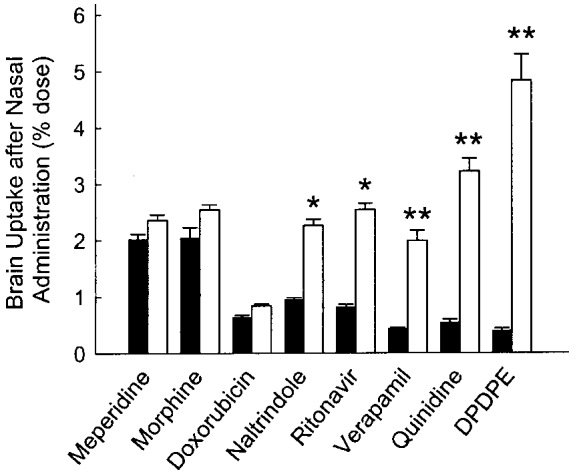

Fig. 2. Brain uptake (5 min) of selected P-gp substrates in *mdr1a(+/+)* (closed bars) and *mdr1a(–/–)* (open bars) mice. Data represent mean ± SD (n = 4), *p < 0.005, **p < 0.001 vs. *mdr1a(+/+)*.

determined over 5 min. The results of this experiment demonstrated a substantial difference in brain uptake between the P-gp-competent and P-gp-deficient mice for P-gp substrates: a 3.1-fold difference for ritonavir, a 4.5-fold difference for verapamil, a 5.9-fold difference for quinidine and a 12.1-fold difference for DPDPE.

Chemical Inhibition of P-gp via Nasal Administration

An additional experiment was performed to evaluate the effect of co-administration of rifampin (500 μM; 5 μL per nostril), an acute P-gp inhibitor, on the delivery of the model P-gp substrate ^{3}H-verapamil to the brain. Control P-gp-competent mice received only methanol as vehicle, and P-gp-deficient mice were used to determine uptake in the complete absence of P-gp-mediated transport. Pre-administration of rifampin resulted in > 4-fold increase in total brain uptake of ^{3}H-verapamil (Fig. 3). In addition, the total brain uptake of ^{3}H-verapamil in animals treated with 500 μM rifampin approximated the total brain uptake observed in *mdr1a(–/–)* mice. To elaborate fully the ability of rifampin to inhibit P-gp-mediated flux via the nasal route, a dose–response relationship was constructed for the influence of rifampin on the brain uptake of ^{3}H-verapamil. The relationship between transport inhibition and rifampin concentration is shown in Fig. 4. The profile was associated with an E_{max} of 99 ± 3% (i.e., essentially complete inhibition of P-gp-mediated transport of ^{3}H-verapamil at administered rifampin concentrations approaching 1 mM), and an ED_{50} of 81 ± 5 μM.

To further elucidate the inhibitory capacity of rifampin on P-gp, a second set of experiments was performed to establish a dose–response curve for rifampin-associated increases in the brain uptake of ^{3}H-quinidine. The relationship between transport inhibition of ^{3}H-quinidine and rifampin concentration (Fig. 5) was characterized by an E_{max} of 53 ± 2%, and an ED_{50} of 112 ± 4 μM.

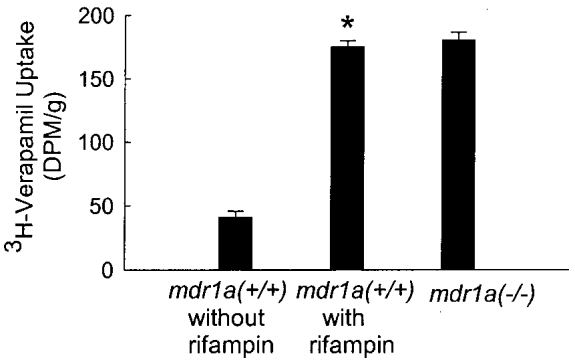

Fig. 3. Reversal of P-gp-mediated exclusion of ^{3}H-verapamil from the brain 3 min after nasal administration. Rifampin (500 μM, 5 μL/nostril) was administered 10 min before ^{3}H-verapamil (5 μM, 2.5 μL/nostril). Data represent mean ± SD (n = 5), *p < 0.001 vs. control (*mdr1a(+/+)* without rifampin). Data from *mdr1a(–/–)* mice included to indicate ^{3}H-verapamil brain uptake in the absence of P-gp-mediated transport.

DISCUSSION

The results of the present studies indicate that the BBB efflux transporter P-gp is functional during nasal administration and serves to limit the total brain uptake of substrates delivered via this route. Most of the studies conducted to date evaluating the nasal route as a means of delivery to the CNS have involved compounds that generally are not substrates for efflux transporters at the BBB. Also, these efforts have used CSF concentrations as an indicator of brain penetration (16). Alternatively, the potential influence of efflux transporters at this barrier has not been explored fully.

This observation was not predicted by the prevailing theory that nasal delivery can serve as a means to circumvent the BBB (20). Instead, it appears that the BBB, including relevant efflux transport systems, is operative at this site. It has been proposed that intranasal delivery provides a means for targeting agents to the brain when those agents do not cross the BBB (21). While there are some mechanisms involved in nasal administration that do allow selected compounds to enter the CNS via the olfactory nerve and thereby

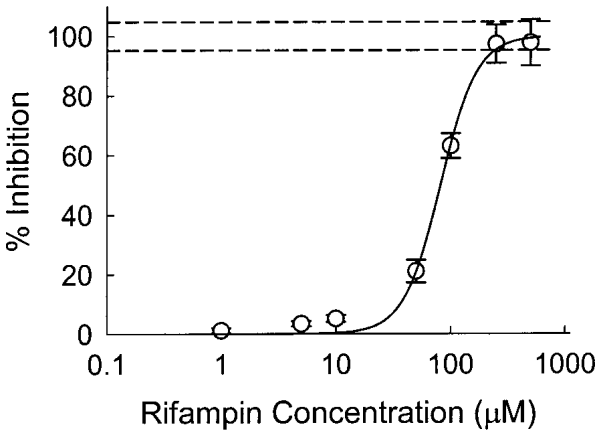

Fig. 4. Dose–response relationship for inhibition of P-gp-mediated efflux transport of ^{3}H-verapamil by rifampin. Symbols represent mean ± SD for n = 4 per rifampin dose. The fitted line represents a sigmoidal Hill equation with E_{max} = 99 ± 3%, ED_{50} = 81 ± 5 μM, γ = 2.7 ± 0.4 (parameter estimate ± standard error). Dashed lines indicate mean ± SD for *mdr1a(–/–)* control mice to document 100% inhibition of P-gp-mediated transport and indicate that there is complete inhibition of P-gp-mediated ^{3}H-verapamil transport with rifampin.

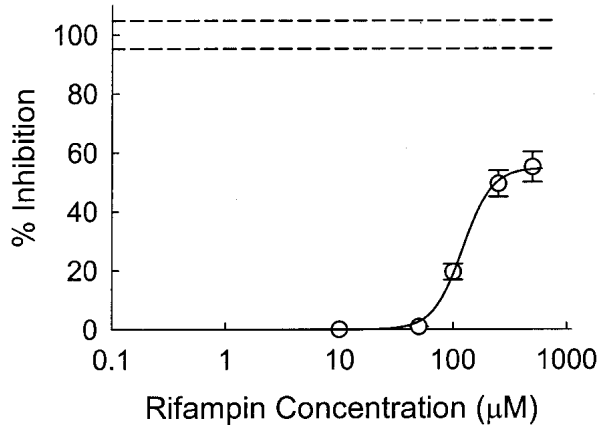

Fig. 5. Dose-response relationship for inhibition of P-gp-mediated efflux transport of ^{3}H-quinidine by rifampin. Symbols represent mean ± SD for n = 4 per rifampin dose. The fitted line represents a sigmoidal Hill equation with E_{max} = 53 ± 2%, ED_{50} = 112 ± 4 μM, γ = 4.9 ± 0.5 (parameter estimate ± standard error). Dashed lines indicate mean ± SD for *mdr1a(–/–)* control mice to document 100% inhibition of P-gp-mediated transport and indicate incomplete inhibition of P-gp-mediated transport of ^{3}H-quinidine with rifampin.

bypass the BBB (22), it also is possible to deliver compounds directly to the brain via nasal instillation without utilizing the olfactory nerve pathway (2). The present results indicate that this direct route may be limited by the action of P-gp and, by analogy, potentially other efflux transporters.

The nasal cavity shares many common features with the BBB, including the presence of tight junctions (23) and the expression of transport proteins and metabolic enzymes (24). Specifically, tight junctions are observed in both the nasal mucosa and olfactory epithelium. Also, there is significant expression of a series of CYP isoforms, including CYP1A2, 2A, 2B, 2C, 2E, and 3A (25,26), and a variety of metabolic enzyme systems, including NADPH-cytochrome P450 reductase, epoxide hydrolase, glucuronosyltransferase and glutathione transferase have shown significant activity in the nasal cavity (27,28). Finally, both P-gp and MRP1 have been documented in the nasal mucosa (29). The potential expression, and the role of, MDR-related transporters in the olfactory epithelium are currently unknown.

The rapid appearance of substrates in brain tissue in these studies indicates that the olfactory epithelial "direct route" is the likely pathway being followed. Furthermore, it is clear that P-gp attenuates the accumulation in brain of intranasally-administered P-gp substrates. In fact, the results of these experiments indicate that the degree of difference in brain uptake after nasal instillation between *mdr1a(+/+)* and *mdr1a(–/–)* mice corresponds to the degree to which P-gp limits brain uptake of that compound after systemic administration. For instance, P-gp plays a limited role in attenuating the brain uptake of morphine during *in situ* brain perfusion in mice (19), and there was a corresponding modest difference in brain uptake after nasal administration. On the other hand, P-gp plays a pivotal role in limiting the brain uptake of compounds such as verapamil and quinidine (19) after systemic administration. P-gp clearly had a major influence on the brain uptake of these compounds after nasal administration (Fig. 3), with a substantial difference in brain uptake between P-gp competent and P-gp-deficient mice (4- and 6-fold, respectively). The limited scope of these preliminary experiments does not allow us to rule out the influence of systemic delivery via the nasal route; however, the short time course of the experiments indicates that there is likely a direct uptake route involved. Thus, biochemical components of the BBB may influence brain penetration of substrates after nasal administration.

The fact that co-administration of a P-gp inhibitor attenuated the barrier function of this efflux transporter, resulting in enhanced delivery of P-gp substrates to the brain via the nasal route, was especially intriguing. These observed transporter barrier functions, regardless of their anatomic location, are reversible. In addition, these experiments indicated that rifampin is able to abolish completely the ability of P-gp to limit the brain uptake of ^{3}H-verapamil, but could inhibit only partially the P-gp-mediated transport of ^{3}H-quinidine. These results agree with previous *in situ* brain perfusion studies performed in this laboratory (30). The underlying mechanism for this substrate-dependent difference is unknown, but may be related to the presence of multiple recognition sites with differing substrate specificities on P-gp (31).

Because rifampin is a substrate for both CYP3A4 and P-gp, it is possible that rifampin-associated changes in CYP3A-mediated metabolism in the olfactory mucosa may have influenced the apparent degree of brain uptake. The design of the experiments reported herein did not address this potential interaction. However, since the maximum inhibition of ^{3}H-verapamil transport by rifampin resulted in brain uptake that was similar to ^{3}H-verapamil uptake in P-gp-deficient mice, it is likely that the effects mediated by rifampin are at the level of transport rather than metabolism.

In summary, rapid penetration into the brain of a variety of compounds was demonstrated after nasal instillation. Brain penetration was modulated by P-gp, and the degree of modulation corresponded to the known effects of P-gp at the blood–brain interface. Efflux transport by P-gp also was reversed by nasal administration of a P-gp inhibitor. Further experimentation is required to fully define the role of P-gp and other efflux transport systems in determining brain uptake after nasal administration, as well as to elucidate the mechanism(s) involved in substrate flux from the nasal cavity to the brain.

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