Morphine Antinociception Is Enhanced in *mdr1a* Gene-Deficient Mice

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Purpose. Previous studies have suggested that P-glycoprotein (P-gp) modulates opioid antinociception for selected μ-and δ-agonists. This study was undertaken to assess morphine antinociception in mice lacking the *mdr1a* gene for expression of P-gp in the CNS.

Methods. Morphine (n = 4–5/group) was administered as a single s.c. dose to *mdr1a*(−/−) mice (3–5 mg/kg) or wild-type FVB controls (8–10 mg/kg). Tail-flick response to radiant heat, expressed as percent of maximum response (%MPR), was used to determine the antinociceptive effect of morphine. Concentrations in serum, brain tissue, and spinal cord samples obtained immediately after the tail-flick test were determined by HPLC with fluorescence detection. Parallel experiments with R(+)-verapamil, a chemical inhibitor of P-gp, also were performed to further investigate the effect of P-gp on morphine-associated antinociception.

Results. Morphine-associated antinociception was increased significantly in the *mdr1a*(−/−) mice. The ED_{50} for morphine was > 2-fold lower in *mdr1a*(−/−) (3.8 ± 0.2 mg/kg) compared to FVB (8.8 ± 0.2 mg/kg) mice. However, the EC_{50} derived from the brain tissue was similar between the two mouse strains (295 ng/g vs. 371 ng/g). Pretreatment with R(+)-verapamil produced changes similar to those observed in gene-deficient mice. P-gp does not appear to affect morphine distribution between spinal cord and blood, as the spinal cord:serum morphine concentration ratio was similar between gene-deficient and wild-type mice (0.47 ± 0.03 vs. 0.56 ± 0.04, $p > 0.05$).

Conclusions. The results of this study are consistent with the hypothesis that P-gp attenuates the antinociceptive action of morphine by limiting the brain:blood partitioning of the opioid.

KEY WORDS: P-glycoprotein; *mdr1a*(−/−) mice; morphine; antinociception; blood–brain barrier; pharmacodynamics.

INTRODUCTION

P-glycoprotein (P-gp) is an ATP-dependent drug efflux transporter encoded by the *mdr1* gene. P-gp is expressed in multidrug resistant (MDR) tumor cells and in several normal tissues, including intestinal epithelial cells, canalicular membranes of hepatocytes, and brain capillary endothelium (1,2). While functions of P-gp in normal tissues have not been well established, its localization suggests that the transporter conveys general protective/excretory characteristics (3).

A small but convincing body of evidence indicates that opioids are substrates for P-gp. The CNS effects of loperamide were higher in *mdr1a*(−/−) than *mdr1a*(+/+) mice, with brain:blood concentration ratios ~6-fold higher in gene-deficient mice than in controls (4). Brain concentrations of asimadoline, a newly developed selective κ-opioid agonist, were ~10-fold higher in *mdr1a*(−/−) mice than in wild-type mice after i.v. administration (5). Recently, several studies have demonstrated that morphine is a P-gp substrate. GF120918, a specific inhibitor of P-gp, has been shown to increase the antinociceptive action of morphine in rats (6,7). Accumulation of morphine in cultured primary bovine brain microvessel endothelial cells (BBMECs) was significantly enhanced by P-gp inhibitors GF120198 and verapamil (8). In addition, morphine accumulation in Chinese hamster ovary cells which overexpress P-gp was lower than in their drug-sensitive counterparts (9), and *mdr1a*(−/−) mice exhibited higher radioactivity in brain compared to wild-type mice (~1.7-fold) after an i.v. dose of [^{3}H]morphine (10).

Previous studies in this laboratory with [D-penicillamine^{2,5}]enkephalin (DPDPE), the first highly selective peptidic δ-opioid receptor agonist, support the hypothesis that opioids are substrates of P-gp. Despite its favorable metabolic stability, DPDPE displays a low blood-brain barrier (BBB) permeability and short residence time *in vivo* (11–13). Extensive *in vitro* and *in vivo* experiments have shown that P-gp-mediated efflux at the BBB limits the magnitude and duration of action of DPDPE (14, 15). Brain, but not plasma, concentrations of DPDPE were increased significantly in *mdr1a*(−/−) mice compared to controls. However, response per unit brain concentration also was much higher (~12-fold) for *mdr1a*(−/−) mice, suggesting that mechanisms other than differences in whole-organ accumulation between the two mouse strains must be responsible in part for observed differences in antinociception.

Calcium channel blocker verapamil is a competitive inhibitor of P-gp (16). Its transport across the BBB involves both passive diffusion and active efflux by P-gp. Although mice lacking P-gp encoded by *mdr1a* gene represent an important *in vivo* model to examine morphine and P-gp interaction at the BBB, studies with chemical inhibitor of P-gp (e.g., verapamil) are necessary to validate the results obtained from *mdr1a* gene-deficient mice.

The purpose of the present study was to determine whether the pharmacodynamics of morphine, an opioid of differing structure from DPDPE and one that acts via a different receptor system (μ-opioid receptor), are modulated by P-gp in a manner similar to DPDPE.

METHODS

Materials

Morphine sulfate and nalorphine hydrochloride were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents used in this study were of the highest grade available from commercial sources.

Animals

Male FVB (wild-type) and *mdr1a*(−/−) mice (4–6 weeks of age) were purchased from Taconic (Germantown, NY). Mice were housed individually in wire-mesh cages in a temperature- and humidity-controlled room with a 12-hr dark/12-hr light cycle. The mice had free access to food and water, and were

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acclimated at least a week prior to experimentation. All procedures were conducted according to the "Principles of Laboratory Animal Care" (NIH publication #85-23).

Assessment of Antinociception

The tail-flick test was used to quantitate antinociceptive response. Tail-flick latency was measured in duplicate with a hot lamp tail-flick analgesia meter (Model 0570-001L, Columbus Instruments International Corp., Columbus, OH). A point 1.5 cm from the distal end of the tail was exposed to the lamp, and lamp intensity was adjusted to produce a baseline latency of 2–3 sec. A cut-off test latency (10 sec) was used to avoid tissue damage. Antinociception was expressed as percent of maximum possible response (%MPR):

$$\%\ \text{MPR} = \frac{\text{Test latency} - \text{Baseline latency}}{\text{Cutoff latency} - \text{Baseline latency}} \bullet 100$$

Morphine Dose-Response Relationship in *mdr1a*(−/−) vs. Control Mice

Morphine (n = 5/dose group) was administered as a single s.c. dose to *mdr1a*(−/−) mice (3–5 mg/kg) or wild-type (FVB) controls (8–10 mg/kg). Tail-flick latency was determined prior to, and at 30 min after, morphine administration. Immediately after testing, mice were sacrificed by decapitation for collection of blood, brain and spinal cord tissue.

Influence of R(+)-Verapamil on Morphine-Associated Antinociception

Mice were assigned randomly to two groups: R(+)-verapamil plus morphine or saline plus morphine. Morphine was administered as a single s.c. dose (3–5 mg/kg for R(+)-verapamil group; 8–10 mg/kg for saline group; n = 5/dose group) 30 min after administration of R(+)-verapamil (100 mg/kg, i.p.) or saline (0.2 ml). Tail-flick latency was determined prior to, and at 30 min after, morphine administration. Immediately after testing, mice were sacrificed by decapitation for collection of blood and brain tissue.

Morphine Assay

All samples were stored at −20°C prior to analysis. Morphine concentrations in serum were determined by HPLC with fluorescence detection modified from the procedures described by Venn *et al.* (17) and Ouellet *et al.* (18). After addition of internal standard (nalorphine 10 μl, 100000 ng/ml), liquid-liquid extraction of alkalinized (pH 9.3) serum (50–100 μl) was performed with chloroform (2 volumes × 2). The organic phase was evaporated to dryness, reconstituted with chloroform (200 μl) and back-extracted with 0.1 M phosphoric acid (120 μl), which was injected on-column. Chromatographic separation was achieved on a 5-μm C6 column and constant-flow (1 ml/min) gradient elution. Solvent A was 14% acetonitrile in 0.1% TFA and solvent B was 0.1% TFA aqueous solution. The initial condition was 50% B (i.e., 7% acetonitrile), which was kept constant for 14 min after sample injection, then ramped down to 0% B (i.e., 14% acetonitrile) over 1 min and remained at 0% B through 24 min. The gradient returned to the initial condition over 1 min, and the column equilibrated for 5 min before injection of the next sample. The fluorescence detector was set at excitation and emission wavelengths of 220 nm and 320 nm, respectively. Morphine and the internal standard eluted at 12 and 22 min, respectively. Standard curves were linear up to 5000 ng/ml, with a limit of quantification of 50 ng/ml when 100 μl serum was extracted.

Whole brain was isolated, blotted dry, weighed and homogenized (1:2 w/v in saline). Aliquots of homogenate were prepared in the same manner as serum samples.

Spinal cord was isolated, blotted dry, weighted and pooled together for each dose group due to the limited amount of available tissue. Pooled tissue was homogenized with saline (1:3 w/v). Aliquots of homogenate were prepared in the same manner as serum samples.

Statistical Analysis

ED_{50} and EC_{50} values were determined by nonlinear least-squares regression (WINNONLN, SCI, Apex, NC). Data are presented as mean ± SE. Analysis of variance (ANOVA) and Student's t-test, where appropriate, were used to determine the statistical significance of differences between experimental groups. In all cases the tests were two-tailed and the criterion for statistical significance was $p < 0.05$.

RESULTS

Morphine Antinociception in *mdr1a*(−/−) Mice

The dose-response relationships in the two mouse strains were approximately parallel (Fig. 1). However, the ED_{50} for morphine was >2-fold lower in *mdr1a*(−/−) mice compared to FVB mice (3.8 ± 0.2 mg/kg vs. 8.8 ± 0.2 mg/kg). The relationship between antinociception and brain tissue concentration was approximately log-linear (Fig. 2). The EC_{50} was slightly higher in FVB mice than gene-deficient mice (371 ng/g and 295 ng/g for FVB and *mdr1a*(−/−) mice, respectively), although this difference is of doubtful pharmacologic significance.

The brain:serum concentration ratio, averaged among all administered doses (Fig. 3), was significantly higher in *mdr1a*(−/−) mice as compared with FVB mice (0.45 ± 0.03 vs. 0.26 ± 0.01, $p < 0.001$). However, no significant differences were observed across dose level in either strain (data not shown),

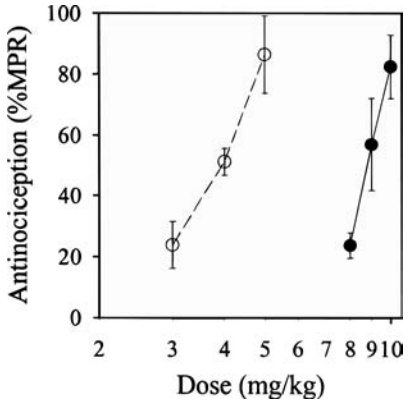

Fig. 1. Antinociception vs. morphine dose in *mdr1a*(−/−) mice (○) and FVB mice (•). Data are presented as mean ± SE.

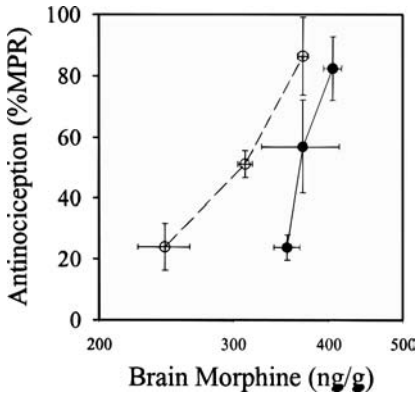

Fig. 2. Antinociception vs. brain morphine in *mdr1a*(−/−) mice (○) and FVB mice (•). Data are presented as mean ± SE.

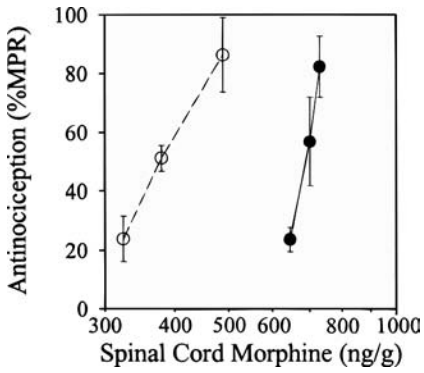

Fig. 4. Antinociception vs. spinal cord morphine in *mdr1a*(−/−) mice (○) and FVB mice (●). Data are presented as pooled average from 4–5 animals per dose group.

suggesting that saturation of transport did not occur at the dose used in this experiment.

The estimated spinal cord EC_{50} for morphine (Fig. 4) was about 2-fold lower in *mdr1a*(−/−) mice compared to FVB mice (381 ng/g vs. 684 ng/g). Unlike brain:serum concentration ratio, no statistically significant difference was observed for the spinal cord:serum concentration ratio in *mdr1a*(−/−) mice and FVB mice (0.47 ± 0.03 vs. 0.56 ± 0.04, $p > 0.05$).

Influence of R(+)-Verapamil on Morphine-Associated Antinociception

R(+)-verapamil shifted the morphine dose-response curve to the left (Fig. 5). The ED_{50} for morphine was >2-fold lower in mice receiving R(+)-verapamil compared to controls (4.0 ± 0.2 mg/kg vs. 8.8 ± 0.2 mg/kg), and was similar to the ED_{50} observed in *mdr1a* gene-deficient mice. The effect of R(+)-verapamil on the relationship between antinociception and brain tissue morphine concentration also was similar to the influence of lack of P-gp in *mdr1a*(−/−) mice (Fig. 6). The EC_{50} values were comparable between two experimental groups (312 ng/g and 324 ng/g for mice receiving R(+)-verapamil and controls, respectively). The brain:serum concentration ratio was significantly higher in R(+)-verapamil-treated mice compared to controls (0.52 ± 0.04 vs. 0.36 ± 0.02, $p < 0.001$), and was similar to the ratio observed in *mdr1a*(−/−) mice (Fig. 3).

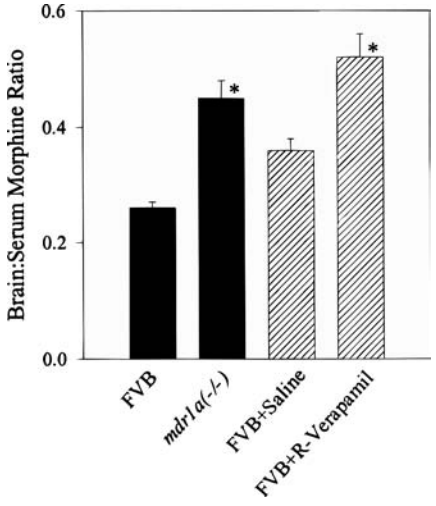

Fig. 3. Influence of P-gp on the mean (± SE) brain:serum morphine concentration ratio. The closed bar represents data obtained from *mdr1a*(−/−) mice; and the hatched bar represents data obtained from mice treated with R(+)-verapamil. *Statistically different from control (FVB for *mdr1a*(−/−); FVB + Saline for FVB + R(+)-verapamil), $p < 0.001$.

DISCUSSION

P-gp is a transmembrane glycoprotein expressed in multidrug resistant tumor cells and several normal tissues (1,3). Although the precise physiologic function of P-gp is not well established, P-gp located in brain capillary endothelium functions as a component of the BBB (4). Previous studies have indicated that mechanisms other than differences in brain accumulation between *mdr1a*(−/−) mice and controls must be partly responsible for observed differences in DPDPE antinociception (14,15). The present study was designed to determine whether morphine behaves in a manner similar to DPDPE.

Morphine-associated antinociception was increased significantly in *mdr1a*(−/−) mice (ED_{50} of 3.8 ± 0.2 mg/kg vs. 8.8 ± 0.2 mg/kg). However, unlike DPDPE, the EC_{50} derived

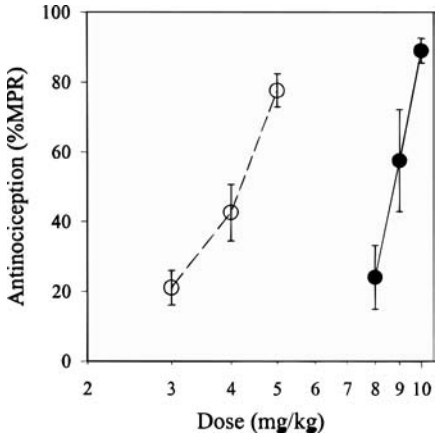

Fig. 5. Antinociception vs. morphine dose in R(+)-verapamil treated mice (○) and saline-treated controls (●). Data are presented as mean ± SE.

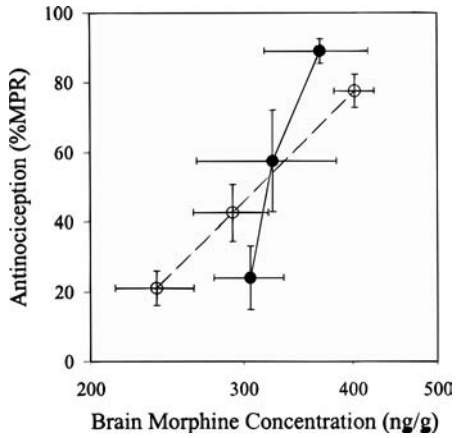

Fig. 6. Antinociception vs. brain morphine concentration in R(+)-verapamil treated mice (○) and saline-treated mice (●). Data are presented as mean ± SE.

from brain tissue was similar between gene-deficient and wild-type mice (295 ng/g vs. 371 ng/g). The brain:serum morphine concentration ratio was approximately 2-fold higher in *mdr1a*(−/−) mice as compared to controls, consistent with the hypothesis that P-gp transports morphine from brain to blood, decreasing morphine concentration at the site of action and attenuating morphine antinociception.

The action of morphine in CNS involves both supraspinal and spinal sites (19). Alteration of either blood-brain or blood-spinal penetration of morphine may affect the degree of antinociception. P-gp has been identified in the epithelial of choroid plexus (20). It is possible that P-gp regulates flux at the blood-spinal barrier differently than flux at BBB, resulting in a different degree of supraspinal/spinal synergy. However, our results suggest that P-gp does not modulate spinal cord morphine concentrations to a significant extent. Spinal cord:serum concentration ratios were similar between *mdr1a*(−/−) mice and controls (0.47 ± 0.03 vs. 0.56 ± 0.04, $p > 0.05$).

While genetically altered animals represent an important tool to study the interaction between compounds and transporters, it must be realized that unrecognized biochemical or physiologic changes may be associated with genetic alterations. Studies with chemical inhibitors of P-gp provide further supporting evidence for the influence of P-gp on blood-brain disposition and antinociception of morphine. Since R(+)-verapamil is 10-fold less potent than the S(−)-isomer in terms of cardiovascular effect (21), R(+)-verapamil was used as a P-gp modulator in the present studies. Preliminary experiments indicated that a 100-mg/kg i.p. dose of R(+)-verapamil was well tolerated by FVB mice and that R(+)-verapamil alone did not exert any antinociceptive effect (data not shown). Consistent with results from *mdr1a*(−/−) mice, significantly higher morphine antinociception was observed in mice treated with R(+)-verapamil (ED_{50} of 4.0 ± 0.2 mg/kg and 8.8 ± 0.2 mg/kg for R(+)-verapamil-treated group and controls, respectively). The impact of treatment with a 100-mg/kg i.p. dose of R(+)-verapamil on accumulation of morphine in brain was comparable to the lack of the *mdr1a* gene (brain:serum concentration ratio of 0.52 ± 0.04 for R(+)-verapamil treated mice vs. 0.45 ± 0.03 for *mdr1a*(−/−) mice, $p > 0.05$). However, unlike previous reports for the δ-receptor agonist DPDPE, neither *mdr1a* gene deficiency nor chemical inhibition of P-gp influenced the EC_{50} of morphine in brain tissue.

Taken together, the results of these experiments suggest that P-gp regulates morphine distribution across the BBB. Unlike DPDPE, P-gp does not appear to affect the relationship between antinociception and brain tissue opioid concentration.

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