**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 and short residence time studies have shown that P-gp-mediated efflux is significant in the CNS and 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 GF120918 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 (1–1.7-fold) after an i.v. dose of [1H]morphine (10).

**Results.** Morphine-associated antinociception was increased significantly in the mdr1a(-/-) mice. The ED₅₀ 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 EC₅₀ derived from the brain tissue was similar between the two mouse strains (295 ng/g vs. 371 ng/g). Pretreatment with R(+)-verapamil, a chemical inhibitor of P-gp, also were performed to further investigate the effect of P-gp on morphine-associated antinociception.

**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 GF120918 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 [1H]morphine (10).

Previous studies in this laboratory with [D-penicillamine]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 was also 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...
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):

\[
\text{% MPR} = \frac{\text{Test latency} - \text{Baseline latency}}{\text{Cutoff latency} - \text{Baseline latency}} \times 100
\]

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, 10000 ng/ml), liquid-liquid extraction of alkalinized (pH 9.3) serum (50–100 μl) was performed with chloroform (2 volumes x 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). 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

ED50 and EC50 values were determined by nonlinear least-squares regression (WINNONLIN, 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 ED50 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 EC50 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).