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Differential Involvement of P-Glycoprotein (ABCB1) in Permeability, Tissue Distribution, and Antinociceptive Activity of Methadone, Buprenorphine, and Diprenorphine: *In Vitro* and *In Vivo* Evaluation

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Abstract

Conclusions based on either *in vitro* or *in vivo* approach to evaluate the P-gp affinity status of opioids may be misleading. For example, *in vitro* studies indicated that fentanyl is a P-gp inhibitor while *in vivo* studies indicated that it is a P-gp substrate. Quite the opposite was evident for meperidine. The objective of this study was to evaluate the P-gp affinity status of methadone, buprenorphine and diprenorphine to predict P-gp-mediated drug-drug interactions and to determine a better candidate for management of opioid dependence. Two *in vitro* (P-gp ATPase and monolayer efflux) assays and two *in vivo* (tissue distribution and antinociceptive evaluation in *mdr1a/b* (–/–) mice) assays were used. Methadone stimulated the P-gp ATPase activity only at higher concentrations, while verapamil and GF120918 inhibited its efflux ($p < 0.05$). The brain distribution and antinociceptive activity of methadone were enhanced ($p < 0.05$) in P-gp knockout mice. Conversely, buprenorphine and diprenorphine were negative in all assays. P-gp can affect the PK/PD of methadone, but not buprenorphine or diprenorphine. Our report is in favor of buprenorphine over methadone for management of opioid dependence. Buprenorphine most likely is not a P-gp substrate and concerns regarding P-gp-mediated drug-drug interaction are not expected.

Keywords

ADME; P-glycoprotein; transporters; distribution; pharmacokinetics; pharmacodynamics

INTRODUCTION

The blood–brain barrier (BBB) acts as a regulator of the flux of xenobiotics between the systemic circulation and the central nervous system (CNS). The permeability of drugs across the BBB has been traditionally considered as a factor of molecular weight, protein binding, H-bonding, degree of ionization and lipid solubility.¹ However, a convincing body of evidence suggests that the efflux transporter, P-glycoprotein (P-gp, Abcb1), may also modulate drug disposition into the brain.^{2–7} P-gp, was the first described MDR transporter, it is a 170 kDa transmembrane protein that is expressed in many tissues such as brain, liver,

spleen, intestine, testes, and kidney. P-gp is encoded by *mdr1a* and *mdr1b* genes in rodents and MDR1 and MDR3 genes in humans and is known to be involved in regulating the disposition of many therapeutic agents including opioids.^{8,9} P-gp may not only have a direct impact on the pharmacological actions of opioids but may also be the locus of opioid-related drug interaction.^{10,11}

Opioid analgesics are by far the most appropriate medication for management of moderate to severe pain, especially cancer and postoperative related pain.¹² Many opioids are P-gp substrates.^{9,10,13–19} P-gp was demonstrated to modulate their absorption, CNS penetration, systemic elimination and antinociceptive activities. For example, the P-gp inhibitors GF120918 and verapamil significantly enhanced morphine penetration in bovine brain microvessel endothelial cells (BBMECs).¹⁵ Brain penetration of morphine was enhanced by 1.7-fold in *mdr1a* (–/–) mice.⁴ The antinociceptive activity of morphine was enhanced by twofold in *mdr1a* (–/–) mice when compared to *mdr1a* (+/+) mice.²⁰ Western blot analysis indicated a twofold upregulation of brain P-gp expression in morphine tolerant rats.¹⁴ The antinociceptive activity of morphine, and fentanyl had increased significantly in *mdr1a/b* (–/–) mice compared to wild-type mice.²¹ P-gp had a variable modulation of the brain uptake (nondetectable to 8-fold increase in brain uptake) of μ , δ , and κ receptors agonists in P-gp competent and P-gp deficient mice.⁹ Recently, we reported that P-gp can also affect the CNS distribution of oxycodone and mediate drug–drug interaction between oxycodone and paclitaxel.¹⁰ Where, chronic oxycodone administration resulted in upregulation of P-gp in brain, liver and kidney tissues and hindered the penetration of paclitaxel, the P-gp substrate, in these tissues.¹⁰ Finally, in our laboratory we demonstrated that many meperidine analogs are also P-gp substrates.¹⁸ These aforementioned studies suggest that P-gp can play a significant role in affecting the transport, antinociceptive efficacy, drug–drug interactions and tolerance development to many opioids which may translate into serious implications on pain management. As such, evaluation of the P-gp affinity status (substrate, nonsubstrate, inhibitor) of the currently used opioids, for example, methadone, buprenorphine and diprenorphine can help in better understanding of the shortcomings of these opioids and aid in better management of pain perhaps through coadministration of P-gp inhibitors with opioids that are P-gp substrates.

Both methadone and buprenorphine are currently approved for management of opioid dependence.²² Since many abused opioids are P-gp substrates, their concomitant administration with methadone or buprenorphine may translate into P-gp mediated drug–drug interactions (if methadone or buprenorphine are P-gp substrates). As such, evaluation of the P-gp affinity status of methadone and buprenorphine was important to determine which of them exhibits minimal P-gp interaction. For such opioid with minimal P-gp interaction, concerns regarding the influence of P-gp on its pharmacokinetics, pharmacodynamics, and drug–drug interaction will not be expected; hence, this opioid will be considered a better candidate for management of opioid dependence. On the other hand, information regarding *in vitro* and *in vivo* evaluation of opioid antagonists (e.g., diprenorphine, naloxone, and naltrexone) is insufficient or lacking. Opioid antagonists are commonly used for management of opioid toxicity or opioid addiction. As a result, evaluation of the P-gp affinity status of diprenorphine will help in predicting P-gp mediated drug–drug interactions in case diprenorphine is coadministered with an opioid agonist that is P-gp substrate.

Although many studies focused on studying the P-gp affinity status of opioids, most of these studies were either *in vitro* or *in vivo* studies. As a result, there were some conflicting data concerning the P-gp affinity status of a number of opioids. For example, *in vitro* studies indicated that fentanyl is a P-gp inhibitor²³ while *in vivo* antinociceptive studies indicated that it is a P-gp substrate.²¹ Quite the opposite was evident for meperidine, where *in vitro*

studies indicated that meperidine is a P-gp substrate²⁴ while *in vivo* brain uptake studies and antinociceptive studies indicated that it is not a P-gp substrate.^{9,21} As such, it is clear that results based on one assay whether it is *in vitro* or *in vivo* must be interpreted with caution. The goal of this study was to elucidate the P-gp affinity status of methadone (opioid agonist), buprenorphine (mixed opioid agonist/antagonist), and diprenorphine (opioid antagonist) (Fig. 1). In order to perform a more definitive evaluation of these opioids, both *in vitro* (P-gp ATPase assay, monolayer efflux assay) and *in vivo* (tissue distribution and antinociceptive monitoring in P-gp deficient/competent mice) approaches were utilized.

MATERIALS AND METHODS

Drug-Stimulated P-gp ATPase Activity

Drug stimulated P-gp ATPase activity was estimated by Pgp-GIO assay system (Promega, Madison, WI). This method relies on the ATP dependence of the light-generating reaction of firefly luciferase. ATP consumption is detected as a decrease in luminescence. In a 96 well plate, recombinant human P-gp (25 μ g) was incubated with P-gp-GIO assay bufferTM (20 μ L) (control, $n = 4$), verapamil (200 μ M) ($n = 4$), sodium orthovanadate (100 μ M) ($n = 4$), methadone (5–100 μ M) ($n = 3/\text{conc.}$), buprenorphine (5–100 μ M) ($n = 3/\text{conc.}$) and diprenorphine (5–100 μ M) ($n = 3/\text{conc.}$). Verapamil served as a positive control while sodium orthovanadate was used as a P-gp ATPase inhibitor. In the presence of sodium orthovanadate ATP consumption by P-gp is negligible and without sodium orthovanadate, P-gp consumes ATP to a greater or lesser extent than the control, dependent on the effect of the test compound. The reaction was initiated by addition of MgATP (10 mM), stopped 40 min later by addition of 50 μ L of firefly luciferase reaction mixture (ATP detection reagent) that initiated an ATP-dependent luminescence reaction. Signals were measured 60 min later and integrated for 10 s using Lmax[®] luminometer (Molecular Devices Corporation, Sunnyvale, CA) and converted to ATP concentrations by interpolation from a luminescent ATP standard curve. The rate of ATP consumption (pmol/min/ μ g protein) was determined as the difference between the amount of ATP in absence and presence of sodium orthovanadate (Eqs. 1 and 2). Drug-stimulated P-gp ATPase activity was reported as fold-stimulation relative to the basal P-gp ATPase activity in the absence of drug (control) (Eq. 3).

Data Analysis

ATPase Assay Data Analysis—Basal P-gp activity, test compound stimulated P-gp activity and fold stimulation by a test compound were calculated according to the following equations:

Basal P-gp activity (pmol ATP consumed/ μ g P-gp/min)

$$= \frac{\text{ATP}_{\text{vanadate}} - \text{ATP}_{\text{control}}}{25_{\mu\text{g Pgp}} \times 40_{\text{min}}} \quad (1)$$

Test compound stimulated P-gp activity (pmol ATP consumed/ μ g P-gp/min)

$$= \frac{\text{ATP}_{\text{vanadate}} - \text{ATP}_{\text{compound}}}{25_{\mu\text{g Pgp}} \times 40_{\text{min}}} \quad (2)$$

Fold stimulation by a test compound

$$= \frac{\text{Test compound stimulated Pgp activity}}{\text{Basal Pgp activity}} \quad (3)$$

where, $\text{ATP}_{\text{vanadate}}$ is the number of nonconsumed (total) pmol of ATP in the presence of sodium orthovanadate. $\text{ATP}_{\text{control}}$ is the number of non-consumed pmol of ATP in presence of the assay buffer. $\text{ATP}_{\text{compound}}$ is the number of nonconsumed pmol of ATP in presence of a test compound. Analysis of variance (ANOVA) followed by Dunnett's posthoc test (SigmaStat™ 2.03 statistical package, V2.03, Systat software, Inc., San Jose, CA) was used to determine the statistical significance of the difference between groups. The 0.05 level of probability was used as the criterion of significance. SEM of the fold stimulation was calculated using the delta-method.²⁵

Caco-2 Transport Studies

Caco-2 transport studies were performed to examine the role of P-gp on the *in vitro* transport of [³H] methadone (0.2 μCi/mL equivalent to 13 nM), buprenorphine (100 μM) and [³H] diprenorphine (0.2 μCi/mL equivalent to 7 nM). Methadone and diprenorphine were supplied from NIDA (Bethesda, MD). Buprenorphine, verapamil, and [¹⁴C] mannitol were purchased from Sigma Chemical Co. (St. Louis, MO). P-gp inhibitor, GF120918, was generously donated by Dr. James Polli (School of Pharmacy, UMB). Caco-2 cell lines were obtained from ATCC (Manassas, VA) and used between passages 30 and 50. Experimental supplies were purchased from Fisher Scientific (Fair Lawn, NJ). Caco-2 cells were seeded at a density of 80,000 cells/cm² onto 12-well, 0.4 μm transwell inserts (Coaster® Corning, NY) and grown in the presence of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) for 21–28 days at 37°C, 95% humidity and 5% CO₂. Tight junctions formation was checked by measuring (1) the transepithelial electrical resistance (TEER) in all transwell inserts and (2) the permeability of the paracellular marker, [¹⁴C] mannitol (0.2 μCi/mL) in three representative transwell inserts. P-gp functionality was checked in Caco-2 cells by conducting bidirectional transport studies using the well known P-gp substrate [¹⁴C] paclitaxel (0.2 μCi/mL) (Sigma) in the absence and presence of the P-gp inhibitors (200 μM verapamil and 2 μM GF120918). GF120918 is also a BCRP inhibitor.⁴⁹ Transepithelial permeability studies for all opioids were conducted from apical to basolateral side (*A–B*) (*n* = 3/group) and basolateral to apical side (*B–A*) (*n* = 3/group) for 90 min with and without preincubation with the P-gp inhibitors (200 μM verapamil and 2 μM GF120918) for 30 min at 37°C. Samples were collected from the receiver compartments and stored at –80°C until analyzed. The apparent permeability coefficients (*P*_{app}) (in absence and presence of the P-gp inhibitors) were determined at sink conditions using Eq. (4) while the efflux ratios (*E*) (in absence and presence of the P-gp inhibitors) were determined using Eq. (5).

Determination of Opioids, Mannitol, and Paclitaxel in the Transport Media

Buprenorphine samples were analyzed following a reported method.²⁶ The assay was performed using a mobile phase of 5 mM sodium acetate buffer (pH 3.75) in acetonitrile (2:8, v/v) and a Waters 474 fluorescence detector (excitation 210 nm, emission 352 nm). A flow-rate of 1.2 mL/min at 25°C was used and buprenorphine was eluted at 4.2 min. The assay was linear (*r*² = 0.987) over the tested concentrations (1–17 μg/mL). The chromatographic HPLC system was composed of: (1) Waters 510 solvent delivery system (Waters-Millipore, Milford, MA), (2) 717 Waters autosampler, (3) 3390A Hewlett Packard Integrator Plotter (Hewlett Packard, Avondale, PA), and (4) Waters Symmetry C-18 (4.6 mm × 250 mm) (Waters-Millipore). Radioactive methadone, diprenorphine, mannitol, and paclitaxel samples were analyzed using Beckman Coulter LS 6500 multi-purpose scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

Transport Studies Data Analysis

The apparent permeability, P_{app} , coefficients were calculated using the following equation.

$$P_{app} = \frac{dQ}{dtAC_0} \quad (4)$$

where dQ/dt is the linear appearance rate of mass in the receiver compartment, A is the surface area of the membrane filter, and C_0 is the initial concentration in the donor compartment. The efflux ratio, E , for all compounds without/with the P-gp inhibitors, were calculated using the formula

$$E = \frac{P_{appB \rightarrow A}}{P_{appA \rightarrow B}} \quad (5)$$

where $P_{app} (B \rightarrow A)$ is the permeability from the basolateral to the apical direction (secretory transport) and $P_{app} (A \rightarrow B)$ is the permeability from the apical-to-basolateral direction (absorptive transport). ANOVA followed by Dunnett's posthoc test (SigmaStat™) was used to determine the statistical significance of the difference between groups. The 0.05 level of probability was used as the criterion of significance.

Experimental Animals

Male *mdr1a/b* (−/−) and male FVB *mdr1a/b* (+/+) mice weighing 28 ± 5 g (20–24 weeks of age) were purchased from Taconic Laboratories (Germantown, NY). The mice were housed individually and allowed to acclimate at least two days before the experiment was conducted. Animals were fed chow and water “*ad libitum*” and maintained on a 12-h light/dark cycle. The protocol for the animal studies was approved by the School of Pharmacy, University of Maryland IACUC.

Tissue Distribution of Opioids in *mdr 1a/b* (+/+) and *mdr 1a/b* (−/−) Mice

Mice were divided based on their genetic differences into two groups, *mdr1 a/b* (−/−) group and *mdr1 a/b* (+/+) group ($n = 9/\text{group}$). Each group was divided into three subgroups. Mice in each subgroup were subjected to a single s.c. dose of either [^3H] methadone (120 $\mu\text{Ci/kg}$ equivalent to 0.003 mg/kg), [^3H] buprenorphine (120 $\mu\text{Ci/kg}$ equivalent to 0.002 mg/kg) or [^3H] diprenorphine (120 $\mu\text{Ci/kg}$ equivalent to 0.002 mg/kg). Thirty minutes post dosing, mice were sacrificed by CO_2 asphyxiation and brain, liver, kidney and plasma samples were collected and frozen at -80°C until analyzed.

Determination of Opioids in Brain, Liver, Kidney, and Plasma Samples

Brain, liver, and kidney tissues were harvested, blotted with filter paper to remove blood, weighed and then homogenized with PBS (1 g/2 mL). Tissue solubilizer, Solvable® (1 mL) (Perkin-Elmer, Waltham, MA) was added to the tissue homogenate (300 μL) then incubated at 50°C for 3 h. Thirty percentage hydrogen peroxide (300 μL) was added to the homogenate then samples were incubated at 50°C for 1 h for decolorization. Samples were left to cool down to room temperature then Ultima Gold scintillation cocktail (10 mL) (PerkinElmer) was added to each sample and kept at room temp for 1 h before counting. For plasma samples, 100 μL was added directly to 10 mL of Ultima Gold scintillation cocktail. All tissues and plasma samples were analyzed at the same time using Beckman Coulter LS 6500 multipurpose scintillation counter. All data were presented as mean $\pm$ SEM. Two tailed student's *t*-test (SigmaStat™) was used to determine the statistical significance between groups at $p < 0.05$.

Assessment of the Antinociceptive Effect in *mdr1a/b* (–/–) and *mdr1a/b* (+/+) Mice

The hot plate test was used to determine the antinociceptive effect in P-gp knockout versus P-gp competent mice. Each opioid was administered to two groups of mice [*mdr1a/b* (+/+) and *mdr1a/b* (–/–)] (*n* = 5/group). No mouse in any group received more than one single s.c. dose of any opioid. The doses of the opioids were as follow, 3 mg/kg methadone, 0.1 mg/kg buprenorphine and 5 mg/kg diprenorphine. Antinociceptive effect was monitored at 5, 15, 30, 45, 60, 90, 120, 150, and 180 min postdosing using the hot plate analgesia meter (UGO BASILE Instrument, Varese, Italy).²⁷ Responses were measured in duplicates after placing mice on a 55°C hot plate. Baseline was determined on the day of the experiment right before dosing and occurred within 3–8 s. A cut-off time of 35 s was selected to prevent tissue damage. Time to the first hind-paw response was recorded. The hind-paw response may be either a paw lick or a foot shake, whichever occurs first. Mice that failed to respond within the respective cut-off time were defined as “analgesic.” The percentages of the maximum possible effect (% MPE) were calculated using Eq. (6).

Hot Plate Test

The hot plate latency values were converted to a percentage of the maximum possible effect (% MPE) and plotted against time.^{28,29} The area under the %MPE versus time curve (AUEC) was calculated using the trapezoidal method.

$$\%MPE = \frac{\text{post drug latency} - \text{predrug latency}}{\text{cutoff} - \text{predrug latency}} \times 100 \quad (6)$$

All data were presented as mean ± SEM. ANOVA with repeated measures (SigmaStat™ 2.03 statistical package) was used to determine the statistical significance between groups. The 0.05 level of probability was used as the criterion of significance.

RESULTS

Effect of Modulators on P-gp ATPase Activity

Different concentrations of methadone, buprenorphine, and diprenorphine (Fig. 2) were examined to determine their effects on the P-gp ATPase activity. Each opioid together with a known excess of ATP was incubated with recombinant human P-gp. ATP consumption due to P-gp stimulation by each opioid was detected as a decrease in luminescence, that is, the higher the potency of a compound to stimulate the P-gp ATPase activity, the lower the luminescence signal. The rate of ATP consumption due to basal P-gp ATPase activity was estimated to be 14.91 ± 4.15 (pmol ATP/μg P-gp/min) (data not shown). Verapamil, the positive control, stimulated the rate of ATP consumption by 80.64 ± 3.42 (pmol ATP/μg P-gp/min) (*p* < 0.001) (data not shown) with >5-fold stimulation of the basal P-gp ATPase (Tab. 1). Methadone stimulated the rate of ATP consumption in a concentration dependant manner (Fig. 2A). At low concentrations, the rate of ATP consumption was not significantly different from the drug-free control, however at higher concentration (100 μM); a significant increase in the rate of ATP consumption was observed (Fig. 2A). This biphasic response for P-gp ATPase activation observed for methadone has also been reported for a variety of compounds, for example, oxycodone, promethazine, propafenone, quinidine, dipridamole and digoxin, and indicate that these compounds require threshold concentrations for measurable ATPase activation.^{10,30,31} As such, the fold stimulation of the basal P-gp activity by methadone was significantly different (*p* < 0.05) from the drug-free membranes only at higher concentration (100 μM) (Tab. 1). For both buprenorphine and diprenorphine, no statistically significant differences (*p* > 0.05) were observed for the rates

of ATP consumption or the fold stimulation of the basal P-gp ATPase activity (Fig. 2B and C and Tab. 1).

Caco-2 Transport Studies

To determine the role of P-gp on the transport of methadone, buprenorphine and diprenorphine, bidirectional transport studies from apical to basolateral side and basolateral to apical side were conducted in absence and presence of verapamil or GF120918, the P-gp inhibitors. To ensure the integrity of the tight junctions, only cells with TEER values $>300 \Omega\text{cm}^2$ were used. The permeability of mannitol, the paracellular marker, was estimated to be 52.1 nm/s indicating tight junctions' formation (data not shown). The functionality of the expressed P-gp in Caco-2 cells was determined by using paclitaxel, as a positive control. Paclitaxel had a high efflux ratio of 82.93 ± 4.58 that decreased dramatically to 6.77 ± 0.59 ($p < 0.01$) and 9.09 ± 0.32 ($p < 0.01$) in presence of verapamil and GF120918, respectively (Tab. 2). These results indicate the presence of active P-gp in the monolayers used and demonstrate the ability of verapamil and GF120918 to modulate this P-gp activity. The efflux ratios of methadone was significantly decreased ($p < 0.05$) in the presence of both verapamil and GF120918 (Tab. 2) indicating that methadone permeability across Caco-2 cells is mediated by P-gp. On the other hand, the efflux ratios of both buprenorphine and diprenorphine were not significantly reduced in the presence of verapamil nor GF120918 (Tab. 2) indicating that both of them are not P-gp substrate.

Tissue Distribution of Opioids in *mdr1a/b* (+/+) and *mdr1a/b* (–/–) Mice

To validate the *in vitro* results and to determine the effect of genetic P-gp disruption on the tissue distribution of methadone, buprenorphine and diprenorphine, a series of *in vivo* studies were conducted using *mdr1a/b* (+/+) and *mdr1a/b* (–/–) mice. Results for methadone, buprenorphine and diprenorphine correspond to drug associated radioactivity. No significant difference in plasma concentrations of any opioid was observed between *mdr1a/b* (+/+) and *mdr1a/b* (–/–) mice (data not shown). This suggests that differences in tissue distribution of opioids would be due to changes in the flux (due to absence of P-gp) rather than changes in the plasma concentrations. We observed that the brain uptake of methadone was significantly ($p < 0.05$) enhanced by ~ 3.52 -fold in *mdr1a/b* (–/–) relative to *mdr1a/b* (+/+) mice (Fig. 3A). However, for the liver and kidney tissues there were no significant differences ($p > 0.05$) in the levels of methadone in *mdr1a/b* (–/–) mice versus *mdr1a/b* (+/+) mice (Fig. 3A). For buprenorphine and diprenorphine, the genetic disruption of P-gp in *mdr1a/b* (–/–) mice had no statistically significant effect ($p > 0.05$) on the distribution of these compounds to the brain, liver, or kidney (Fig. 3B and C).

Assessment of the Antinociceptive Effect in *mdr1a/b* (–/–) and *mdr1a/b* (+/+) Mice

The antinociceptive effects associated with single dose administration of methadone, buprenorphine, or diprenorphine were monitored for 180 min in P-gp knockout and P-gp competent mice using the hot plate analgesia test (Fig. 4). The %MPE for methadone peaked at 15 min for both *mdr1a/b* (–/–) and *mdr1a/b* (+/+) mice (Fig. 4A). In addition, the %MPE was found to be significantly higher for the P-gp knockout mice versus wild-type between 45 and 150 min after dosing. While the %MPE for buprenorphine peaked at 45 min for both *mdr1a/b* (–/–) and *mdr1a/b* (+/+) mice (Fig. 4B). For the opioid antagonist, diprenorphine, the %MPEs were not significantly different ($p < 0.05$) from the basal activity for both *mdr1a/b* (–/–) and *mdr1a/b* (+/+) mice throughout the entire experiment (Fig. 4C). The genetic disruption of P-gp resulted in a significant ($p < 0.05$) increase in the anti-nociceptive activity of methadone indicated by ~ 3 -fold increase in the AUEC of the *mdr1a/b* (–/–) mice relative to that of the *mdr1a/b* (+/+) mice (Tab. 3). For buprenorphine and diprenorphine the AUECs were not significantly ($p > 0.05$) different for the *mdr1a/b* (–/–) mice versus the *mdr1a/b* (+/+) mice (Tab. 3).

DISCUSSION

The objective of this study was to evaluate the P-gp affinity status of methadone, buprenorphine and diprenorphine to predict P-gp-mediated drug–drug interactions and to determine a better candidate for management of opioid dependence. Classification of opioids or any other compound as a P-gp substrate, nonsubstrate, inducer or inhibitor based on only one assay may be misleading.^{1,21,23,32} As a result, we used two *in vitro* (P-gp ATPase and monolayer efflux assays) and two *in vivo* (opioids' tissue distribution and antinociceptive evaluation in *mdr1a/b* (–/–) and *mdr1a/b* (+/+) mice) assays to obtain a comprehensive evaluation of the effect of P-gp on the aforementioned opioids. Data from the P-gp ATPase assay and the monolayer efflux assay indicated that methadone is a P-gp substrate (Tabs. 1 and 2). Consistently, *in vitro* studies using rat everted intestinal sac indicated that the P-gp inhibitors verapamil and quinidine can significantly enhance the transport of methadone.³³ Our, *in vivo* studies were in good agreement with the *in vitro* studies and demonstrated that the CNS distribution of methadone is P-gp dependant. There was a ~3.5-fold increase in brain/plasma ratio of methadone in *mdr1a/b* (–/–) mice versus wild-type mice (Fig. 3A). Upon single dose administration of 3 mg/kg methadone to both P-gp deficient and wild-type mice, the antinociceptive activity was enhanced by 2.73-fold in the P-gp deficient mice (Fig. 4A and Tab. 3). As shown in Figure 3, methadone levels were found to be significantly higher in the brain of the *mdr1a/b* (–/–) mice. This suggests that the lack of P-gp in the *mdr1a/b* (–/–) mice facilitates the CNS penetration of methadone, and the higher CNS levels in turn produce an enhanced pharmacodynamic effect.

Our study is the first to provide comprehensive *in vitro* and *in vivo* evaluation of the P-gp effect on methadone and gives strong evidences that the transport, CNS accumulation and antinociceptive activity of methadone are P-gp dependant. Many reports demonstrated that P-gp can mediate drug-drug interactions when two P-gp substrates compete for the same binding site on P-gp or when a P-gp substrate is concomitantly administered with a P-gp inhibitor.^{8,10,11} This indicates that the tissue distribution and the therapeutic efficacy of methadone (P-gp substrate) may be affected when it is concomitantly administered with a competitive P-gp-substrate or -inhibitor. Figure 3A demonstrates that methadone is extensively localized in the liver and kidney tissues of both P-gp deficient and P-gp competent mice but interestingly P-gp ablation in *mdr1a/b* (–/–) mice had no significant ($p > 0.05$) effect on methadone accumulation in these tissues. The extensive accumulation of methadone in the liver and the kidney tissues can be explained by the high tissue binding and the large volume of distribution (2–5 L/kg) that are known for methadone.³⁴ But, it was expected that the lack of P-gp expression in *mdr1a/b* (–/–) mice would enhance the accumulation of methadone in these tissues, apparently, this is not the case (Fig. 3A). One possible explanation may be, for highly perfused tissues such as liver and kidney, rapid flux of P-gp substrates may result in P-gp saturation or insufficient drug concentrations in the inner membrane leaflet leading to masking of the P-gp-mediated efflux. This process is known as futile cycling of the transporter, in which, the influx across the membrane dominates the efflux out of the cells.^{35–37} Another explanation is, the function of P-gp across tissues most likely is un-equivalent and may be substrate, dose and time dependant.^{3,21,38} Consistent with our report, the distribution of many well known P-gp substrates (e.g., digoxin, dexamethasone, cyclosporin A, and morphine) was not significantly ($p > 0.05$) enhanced in the liver and the kidney tissues of P-gp knockout mice relative to wild type mice.^{3,4}

Evaluation of buprenorphine, the opioid agonist/antagonist, using the above four assays indicated that it is not a P-gp substrate. It was not capable of stimulating the P-gp ATPase activity even at higher concentrations (100 μ M) (Fig. 2B and Tab. 1). It had a low efflux ratio (1.28) that was not significantly ($p > 0.05$) altered in presence of verapamil or

GF120918 (Tab. 2). Ablation of P-gp in *mdr1a/b* ($-/-$) mice had no significant ($p > 0.05$) effect on buprenorphine distribution to the brain, liver, or kidney tissues (Fig. 3B). Finally, its antinociceptive effect was not enhanced in P-gp knockout mice evident by nonsignificant difference in its AUEC in P-gp competent and P-gp knockout mice (Fig. 4 and Tab. 3). In a clinical study³⁹ examining the effect of placental P-gp on the transfer of buprenorphine, *L*- α -acetylmethadol (LAAM) (methadone congener) and paclitaxel in absence and presence of the P-gp inhibitor, GF120918, it was demonstrated that P-gp mediated the efflux of LAAM, paclitaxel but not buprenorphine, and the authors concluded that buprenorphine is not a P-gp substrate. These findings are consistent with those reported herein and support the hypothesis that P-gp is unlikely to affect the tissue distribution of buprenorphine. Recently, modest effects of several P-gp inhibitors/substrates (cyclosporin A, quinidine, verapamil, vinblastin, and vincristine) on the brain uptake of buprenorphine were reported in rats.⁴⁰ The effects ranged from no detectable enhancement (vinblastin and vincristine) to only ~1.5-fold enhancement (cyclosporin A, quinidine, and verapamil) of the apparent brain uptake of buprenorphine. Despite the variable effects of these nonspecific P-gp inhibitors, the authors concluded that P-gp may in part mediate the disposition of buprenorphine. Although this might be true for rats, it is unlikely to be the case for mice (Fig. 3B) or humans.³⁹

Safe and effective managements of opioid dependence impose a great therapeutic challenge. Methadone was the first approved opioid agonist for management of opioid dependence.²² Methadone is very effective but its chronic administration may be associated with diversion, dependence and overdose-related deaths that limit its use to specialized clinics. Our report points to an additional limitation regarding methadone usage. Methadone appears to be a P-gp substrate and its permeability to the brain is mediated in part by P-gp (Fig. 3A). Since many drugs of abuse (e.g., morphine, oxycodone, and cocaine) are P-gp substrates, these drugs can compete with methadone for the same binding site on P-gp leading to enhanced CNS-permeability of either methadone or the drugs of abuse. This enhanced permeability may result into serious overdose associated complications. On the other hand, buprenorphine has recently been approved for the management of opioid dependence.²² It is a partial opioid agonist that has a unique ceiling effect and contrary to methadone, it was approved for less restricted office-based use. Our report is in favor of buprenorphine over methadone for treatment of opioid dependence. Buprenorphine does not appear to be a P-gp substrate (Figs. 2–4 and Tab. 2) and concerns regarding P-gp mediated drug–drug interaction and overdose related complications are not expected.

In vitro studies¹ indicated that some opioid antagonists, for example, naloxone and naltrexone are non-P-gp substrates, however, to the best of our knowledge no *in vivo* studies are available regarding the P-gp affinity status of any opioid antagonist. With that in mind, we decided to evaluate whether the opioid antagonist diprenorphine is a P-gp substrate. [¹¹C]Diprenorphine is extensively used as a positron emission tomography (PET) ligand,⁴¹ it binds to μ , κ , and δ opioid receptors and aids in imaging of opioid receptors occupancy by opioid agonists. The data reported herein suggest that diprenorphine is not a P-gp substrate. Over a wide range of concentrations (5–100 μ M) diprenorphine was not capable of stimulating the P-gp ATPase activity (Fig. 2 and Tab. 1). In the monolayer efflux assay, diprenorphine efflux ratio was not significantly decreased in the presence of verapamil or GF120918 (Tab. 2). The efflux ratio remained ~2 in the absence and presence of both verapamil and GF120918 suggesting that diprenorphine may be transported by efflux transporters other than P-gp (e.g., MRP) across Caco-2 cells. *In vivo* tissue distribution studies were consistent with the *in vitro* studies and no statistically significant difference ($p > 0.05$) was observed for diprenorphine distribution in P-gp knockout versus wild type mice (Fig. 3C). Diprenorphine lacks opioid-agonist properties evident by “sodium ratio” and “GTP ratio” of 1.0 which are characteristics of antagonists that exhibit no agonist properties.^{42,43} This explains why no analgesic effect was associated with its use in both P-

gp knockout and wild type mice (Fig. 4C). The majority of opioids are P-gp substrates which upon chronic administration can result in development of tolerance and dependence. Our data suggests that diprenorphine is not a P-gp substrate and in case it is used for management of opioid toxicity or opioid addiction, it is unlikely that P-gp mediated drug-drug interaction will take place.

Knowing that most opioids are P-gp substrates,^{9,10,18,44–50} P-gp can affect their (1) oral absorption, (2) CNS accumulation, (3) systemic clearance, (4) antinociceptive effect, and (5) tolerance development to their analgesic effect. In addition, P-gp can be the locus of drug–drug interactions. We have reported previously that oxycodone is a P-gp substrate; its chronic administration induced the level of expression of P-gp in the brain, liver, kidney and intestine.¹⁰ This induction resulted in P-gp-mediated drug–drug interaction with paclitaxel, the P-gp substrate.¹⁰ We have also demonstrated that other efflux transporters were upregulated in the brain tissues of oxycodone treated rats, for example, Bcrp (Abcg2) and Mrp4 (Abcc4) (unpublished results). Bcrp upregulation adversely hindered the accumulation of mitoxantrone, the Bcrp substrate. These studies support the hypothesis that efflux transporters may not only affect the PK/PD of opioids but also can mediate drug–drug interactions.^{8,11} We believe it is of great therapeutic importance to develop opioids that are non-P-gp substrates. This will increase the analgesic efficacy, decrease tolerance development and decrease P-gp mediated opioid-drug interaction. In this regard, we synthesized and tested the P-gp affinity status of a series of meperidine ($n = 11$)¹⁸ and morphine ($n = 12$) (manuscript under review) analogs, searching for non-P-gp substrates. Although the majority of these opioids were P-gp substrates, we observed that some opioid agonists, for example, meperidine, *N*-phenylbutyl-*N*-normeperidine, 6-desoxycodine, oxymorphone, thevinone, and methylthevinone were non-P-gp substrates. Studying the pharmacophore of these opioids together with buprenorphine can aid in developing a new class of opioids that are non-P-substrates. These new opioids are expected to have better BBB permeability, better antinociceptive activity, delayed development of tolerance, and minimal P-gp-mediated drug–drug interactions.

In summary, the P-gp affinity status of methadone, buprenorphine and diprenorphine were evaluated using *in vitro* and *in vivo* approaches. Data from the four reported assays (P-gp ATPase assay, monolayer efflux assay, tissue distribution and antinociceptive testing in *mdr1a/b* (+/+) versus *mdr1a/b* (–/–) mice) displayed consistent results concerning the P-gp affinity status of the evaluated opioids. Methadone was positive in the four assays indicating that it is an unambiguous P-gp substrate while buprenorphine and diprenorphine were negative in all the four assays suggesting that they are not P-gp substrates. P-gp can affect the PK/PD of methadone but not buprenorphine or diprenorphine. Finally, and in contrast to methadone, P-gp-mediated drug-drug interaction is unlikely to take place if buprenorphine and diprenorphine are concomitantly administered with other therapeutic agents that are P-gp substrates or inhibitors.

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Abbreviations

MDR	multidrug resistance proteins
P-gp	P-glycoprotein

MRP	multidrug resistance-associated proteins
Caco-2	epithelial human colon adenocarcinoma cell line
% MPE	percentage of maximal possible effect
AUEC	area under the percentage maximum possible effect versus time curve
BBMECs	bovine brain microvessel endothelial cells

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Figure 1.
Chemical structure of methadone, buprenorphine, and diprenorphine.

Figure 2.

The rates of ATP consumption by different opioids as determined by Pgp-GIO assay system (Promega). This method relies on the ATP dependence of the light-generating reaction of firefly luciferase where ATP consumption is detected as a decrease in luminescence. Recombinant human P-gp (25 μg) was incubated with methadone (5–100 μM) ($n = 3/\text{conc.}$) (A), buprenorphine (5–100 μM) ($n = 3/\text{conc.}$) (B) and diprenorphine (5–100 μM) ($n = 3/\text{conc.}$) (C). The rates of ATP consumption were calculated using Eqs. (1) and (2) while the fold stimulation of the basal activity by each opioid was calculated using Eq. (3) and presented in Table 1. Data in the represented figure are expressed as the mean $\pm$ SEM ($n = 3-4$). Negative signs on the ordinate indicate inhibitory effects, however these effects were

not significantly different from the basal activity ($p > 0.05$). Significant differences were only observed for the highest concentration of methadone (100 μM). (Asterisk indicating significant difference is not presented).

Figure 3.

Tissue distribution of methadone (A), buprenorphine (B) and diprenorphine (C) in *mdr1 a/b* (–/–) and *mdr1 a/b* (+/+) mice. Mice in each group were subjected to a single s.c. dose of either [^3H] methadone (120 $\mu\text{Ci/kg}$ equivalent to 0.003 mg/kg), [^3H] buprenorphine (120 $\mu\text{Ci/kg}$ equivalent to 0.002 mg/kg) or [^3H] diprenorphine (120 $\mu\text{Ci/kg}$ equivalent to 0.002 mg/kg). Thirty minutes post dosing, mice were sacrificed and opioids distribution into the brain, liver, and kidney tissues were determined. Data are expressed as the mean $\pm$ SEM ($n = 3/\text{subgroup}$). Asterisk (*) indicates significant difference at $p < 0.05$ as determined by the student's *t*-test using SigmaStatTM 2.03 statistical package.

Figure 4.

Hot plate latencies expressed as %MPE (calculated using Eq. 6) versus time for *mdr1a/b* (+/+) mice (■) and *mdr1a/b* (-/-) mice (▲) that received single i.p. dose of 3 mg/kg methadone (A), 0.1 mg/kg buprenorphine (B) or 5 mg/kg diprenorphine (C). No mouse in any group received more than one single s.c. dose of any opioid. Data are expressed as the mean $\pm$ SEM ($n = 5$ /group). The AUEC were calculated and represented in Table 3.

Table 1

Fold Stimulation of Basal P-gp-ATPase Activity by Verapamil (Positive Control) and Opioids

Compound	Concentration (μM)	Stimulation (Fold) ^a
No compound	0	1.00 $\pm$ 0.39
Verapamil	200	5.41 $\pm$ 1.52 ^a
Methadone	5	-1.51 $\pm$ 1.96
	25	0.74 $\pm$ 1.28
	100	2.45 $\pm$ 0.27 ^b
Buprenorphine	5	0.92 $\pm$ 0.34
	25	-1.22 $\pm$ 0.72
	100	-1.08 $\pm$ 0.61
Diprenorphine	5	-0.59 $\pm$ 0.77
	25	-0.94 $\pm$ 0.70
	100	-0.91 $\pm$ 0.96

Values are expressed as mean $\pm$ SEM ($n = 3-4$).

^a Negative sign indicates inhibitory effects [nonsignificantly different ($p > 0.05$) from the control (no compound)].

^b Significantly different from the control (no compound) at $p < 0.05$.

Table 2

Permeability of Paclitaxel (Positive Control) and Opioids Across Caco-2 Cells in Absence and Presence of P-gp Inhibitors

Compound	$P_{app} A-B$ (nm/s)	$P_{app} B-A$ (nm/s)	$B-A/A-B$ Ratio
Paclitaxel	4.94 ± 0.24	409.62 ± 10.34	82.93 ± 4.58
Paclitaxel + verapamil	27.09 ± 2.06^a	183.36 ± 7.59^a	6.77 ± 0.59^a
Paclitaxel + GF120918	20.17 ± 0.53^a	183.24 ± 4.35^a	9.09 ± 0.32^a
Methadone	77.13 ± 1.83	194.46 ± 10.11	2.52 ± 0.14
Methadone + verapamil	134.18 ± 4.62^a	194.05 ± 2.46	1.45 ± 0.05^a
Methadone + GF120918	66.41 ± 7.52	110.24 ± 17.91^a	1.66 ± 0.03^a
Buprenorphine	446.95 ± 36.92	562.35 ± 5.22	1.26 ± 0.10
Buprenorphine + verapamil	666.67 ± 33.24^a	623.76 ± 35.90	0.94 ± 0.07
Buprenorphine + GF120918	477.54 ± 8.71	721.58 ± 2.12^a	1.51 ± 0.03
Diprenorphine	249.36 ± 13.36	527.77 ± 10.81	2.12 ± 0.12
Diprenorphine + verapamil	203.81 ± 5.03^a	490.73 ± 9.56	2.41 ± 0.12
Diprenorphine + GF120918	285.01 ± 9.49	547.42 ± 31.64	1.92 ± 0.13

Values are expressed as mean $\pm$ SEM ($n = 3$).

^aSignificantly different from P-gp inhibitors-untreated cells ($p < 0.05$).

Table 3

Area Under the Effect Curve (AUEC) (% MPE $\times$ min) of Opioids After Single s.c. Dose in *mdr1a/b* (+/+) and *mdr1a/b* (-/-) Mice

Compound	Dose (mg/kg)	<i>mdr1a/b</i> (+/+)	<i>mdr1a/b</i> (-/-)	AUEC Ratio <i>mdr1a/b</i> (-/-)/ <i>mdr1a/b</i> (+/+)
Methadone	3	1501.85 $\pm$ 246.45	4099.26 $\pm$ 488.58 ^a	2.73 $\pm$ 0.55 ^a
Buprenorphine	0.1	1943.74 $\pm$ 326.81	2854.93 $\pm$ 325.27	1.47 $\pm$ 0.3
Diprenorphine	5	139.85 $\pm$ 121.95	179.22 $\pm$ 64.53	1.28 $\pm$ 1.05

AUEC values are expressed as mean $\pm$ SEM ($n = 5$).

^aIndicates significant differences at $p < 0.05$.