P-glycoprotein is a Major Determinant of Norbuprenorphine Brain Exposure and Antinociception

Sarah M. Brown, Scott D. Campbell, Amanda Crafford,
Karen J. Regina, Michael J. Holtzman, Evan D. Kharasch

From the Departments of Pediatrics (S.B.), Anesthesiology (S.C., A.C., K.R. and E.K.), Medicine (M.H.), Biochemistry and Molecular Biophysics (E.K.), Washington University in St. Louis, St. Louis, MO
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Address correspondence to: Dr. Evan D. Kharasch, Department of Anesthesiology, Washington University in St. Louis, 660 S. Euclid Ave, Campus Box 8054, St. Louis, MO 63110-1093, Tel: 314-362-8796, Fax: 314-362-8571, email: kharasch@wustl.edu

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Abbreviations: P-gp, P-glycoprotein; MDR, Multidrug resistance protein; MDCK, Madin-Darby Canine Kidney Cells; FBS, fetal bovine serum; Papp, apparent permeability

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Abstract

Norbuprenorphine is a major metabolite of buprenorphine and potent agonist of mu, delta and kappa opioid receptors. Compared to buprenorphine, norbuprenorphine causes minimal antinociception but greater respiratory depression. It is unknown whether the limited antinociception is due to low efficacy or to limited brain exposure. Norbuprenorphine is an in vitro substrate of the efflux transporter P-glycoprotein (Mdr1), but the role of P-glycoprotein in norbuprenorphine transport in vivo is unknown. This investigation tested the hypothesis that limited norbuprenorphine antinociception results from P-gp mediated efflux and limited brain access. Human P-glycoprotein-mediated transport in vitro of buprenorphine, norbuprenorphine and their respective glucuronide conjugates was assessed using transfected cells. P-glycoprotein-mediated norbuprenorphine transport and consequences in vivo were assessed using mdr1a(+/+) and mdr1a(-/-) mice. Antinociception was determined by hot water tail-flick assay and respiratory effects by unrestrained whole body plethysmography. Brain and plasma norbuprenorphine and norbuprenorphine-3-glucuronide were quantified by mass spectrometry. In vitro, the net P-glycoprotein-mediated efflux ratio for norbuprenorphine was 9, indicating significant efflux. In contrast, the efflux ratio for buprenorphine and the two glucuronide conjugates was unity, indicating absent transport. The norbuprenorphine brain/plasma concentration ratio was significantly greater in mdr1a(-/-) than mdr1a(+/+) mice. The magnitude and duration of norbuprenorphine antinociception were significantly increased in mdr1a(-/-) compared with mdr1a(+/+) mice, whereas the reduction in respiratory rate was similar. Results show that norbuprenorphine is an in vitro and in vivo substrate of P-glycoprotein. P-glycoprotein-mediated efflux influences the brain access and antinociceptive but not the respiratory effects of norbuprenorphine.
INTRODUCTION

The long-duration opioid buprenorphine has been used for several decades for treating acute and chronic pain, and is now also marketed for opioid addiction and withdrawal therapy. Buprenorphine is a partial agonist at the mu opioid receptor and an antagonist at the kappa opioid receptor. Like other mu agonists, it causes analgesia, respiratory depression, miosis and mood changes, but unlike typical mu agonists there is a ceiling effect at higher doses (Cowan et al., 1977; Walsh et al., 1994). Buprenorphine is extensively metabolized in humans, primarily to norbuprenorphine, and both undergo subsequent glucuronidation to buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide (Zacny et al., 1997; Moody et al., 2009). In humans, peak plasma concentrations of norbuprenorphine are similar to or exceed those of buprenorphine. The relative norbuprenorphine, buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide exposures, based on molar area under the plasma concentration versus time curves are, 200%, 100%, and 600% those of buprenorphine (Moody et al., 2002).

Norbuprenorphine is pharmacologically active, and norbuprenorphine formation may be a bioactivation pathway, rather than an inactivation pathway, as originally considered (Ohtani et al., 1995). Norbuprenorphine has high affinity for the mu, delta, and kappa opioid receptors (Huang et al., 2001; Brown et al., 2011). Norbuprenorphine causes marked respiratory depression in animal models (Ohtani et al., 1997; Brown et al., 2011) but has comparatively less antinociceptive effects (Ohtani et al., 1995). In contrast, buprenorphine causes much greater antinociception but has no respiratory effects in animal models (Ohtani et al., 1995). In rats, norbuprenorphine had 1/50th the analgesic potency of buprenorphine but 10-fold greater respiratory depressant potency (Ohtani et al., 1997). Norbuprenorphine-3-glucuronide (kappa, but not mu or delta) and buprenorphine-3-glucuronide (mu and delta, but not kappa) also have affinity for opioid receptors and are biologically active, causing mild respiratory and
antinociceptive effects, respectively (Brown et al., 2011). The role of norbuprenorphine-3-glucuronide in mediating the effects of norbuprenorphine, and norbuprenorphine-3-glucuronide analgesic efficacy and central nervous system access are unknown. The mechanism of limited norbuprenorphine antinociception, as well as that underlying the differences between norbuprenorphine and buprenorphine, is unknown. These have been attributed to either low norbuprenorphine intrinsic analgesic activity, or to limited brain access of norbuprenorphine (Ohtani et al., 1995).

With the exception of essential nutrients, the movement of endogenous and exogenous substances from the blood to the brain is highly restricted by the blood-brain barrier (BBB). The BBB is formed by a unique suite of tight junction proteins, receptors, and transporters expressed by the endothelial cells that make up the microvasculature of the brain (Abbott, 2005; Engelhardt, 2011). Due to the restrictive nature of the BBB the vast majority of molecules that bind with high affinity to one or more receptors in the brain have little biological activity in vivo (Pardridge, 1997). P-glycoprotein (P-gp, multi-drug resistance protein, MDR1, ABCB1) is one of several transmembrane efflux transporters at the BBB that utilize ATP hydrolysis to protect the brain from a wide variety of exogenous and endogenous compounds. P-glycoprotein has a broad substrate profile, has been shown to regulate brain access of numerous drugs via active efflux, and determines the pharmacologic effect of several drugs acting in the central nervous system (Kim et al., 1998; Jolliet-Riant and Tillement, 1999; Boulton et al., 2002; Uhr et al., 2003; Dagenais et al., 2004; Wang et al., 2004; Sasongko et al., 2005).

Several opioids have been reported previously in animals and/or humans to be in vitro or in vivo substrates for P-glycoprotein, including loperamide (Wandel et al., 2002; Skarke et al., 2003), morphine (Xie et al., 1999; Wandel et al., 2002; Dagenais et al., 2004), methadone (Dagenais et al., 2004; Kharasch et al., 2004), fentanyl (Wandel et al., 2002; Dagenais et al., 2004), alfentanil (Wandel et al., 2002; Kalvass et al., 2007), and sufentanil (Wandel et al.,
Norbuprenorphine was recently identified as a substrate for P-gp mediated efflux by \textit{in vitro} screening using transfected Madin-Darby Canine Kidney (MDCK) cells (Tournier et al., 2010).

Mice lacking Mdr1a and/or Mdr1b have been shown to be useful tools for investigating the \textit{in vivo} role of P-gp in pharmacology (Doran et al., 2005; van Waterschoot and Schinkel, 2011). Genetic P-gp knockouts have been compared to chemical P-gp knockouts in several studies, and the results have shown that genetic disruption of the P-gp gene product has the same effect as chemically inhibiting the transporter (Polli et al., 1999; Zong and Pollack, 2000). \textit{Mdr1a}(-/-) mice have been used to determine that P-gp limits opioid-induced analgesia of morphine, methadone, and fentanyl (Thompson et al., 2000; Hassan et al., 2009).

The purpose of the current investigation was to test the hypothesis that limited norbuprenorphine antinociception is the result of P-gp mediated efflux and limited brain access, using \textit{in vitro} and \textit{in vivo} methods. It evaluated P-gp-mediated transport of buprenorphine, norbuprenorphine, buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide \textit{in vitro}, and the antinoceptive and respiratory effects of norbuprenorphine \textit{in vivo}, using \textit{mdr1a} deficient and wild-type mice. This research is significant because it identifies norbuprenorphine as an avid substrate of P-glycoprotein, which influences norbuprenorphine brain access, and it provides evidence for blood-brain barrier transport differentially influencing the classical opioid effects of analgesia and respiratory depression.
MATERIALS AND METHODS

Materials. Buprenorphine, norbuprenorphine and buprenorphine-3-β-D-glucuronide were provided by the National Institute on Drug Abuse. Norbuprenorphine-3-β-D-glucuronide was synthesized as described in Fan et al. (Fan et al., 2011). Buprenorphine, norbuprenorphine, buprenorphine-3-β-D-glucuronide and norbuprenorphine-3-glucuronide used as analytical standards were from Cerrilliant (Round Rock, TX). Dulbecco’s Modified Eagle’s Media (DMEM), heat inactive fetal bovine serum (FBS) and Hank’s Buffered Salt Solution (HBSS) were obtained from MediaTech (Manassas, VA). Bac-off antibiotic was obtained from Cell-Systems, Inc. (Kirkland, WA). Cell transport assays were conducted using the Corning HTS Transwell system (Corning, Lowell, MA). All other reagents were from Sigma Aldrich (St. Louis, MO).

In vitro transport assay. Madin-Darby Canine Kidney cells (MDCK) transfected with the gene encoding the human multidrug resistance protein MDR1 (P-glycoprotein, P-gp) were obtained from P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). Both the untransfected and transfected cell lines were cultured in DMEM containing 10% FBS, and 1x Bac-off antibiotic. Cells were seeded onto the Transwell supports at a density of 49,000 cells/well and incubated for 4 days at 37°C/95% humidity/5%CO₂.

On the day of the assay the media was aspirated and replaced with HBSS supplemented with 1 mM CaCl₂, 0.1 mM MgSO₄, 0.1 mM MgCl₂ (HBSSCM) and allowed to equilibrate for 30 minutes at 37°C. The buffer was then aspirated and replaced with fresh HBSSCM in either the apical (upper) or basolateral (lower) chamber. HBSSCM that contained 2 µM of the test drug was added to the opposite chamber and the cells incubated for 2 hours at 37°C/95% humidity/5%CO₂. The donor and receiver chambers for each well were sampled and analyzed by LC/MS/MS as described below. Monolayer integrity was checked using lucifer yellow. Briefly, 200 µl of a 100 µg/ml lucifer yellow solution in HBSSCM was added to the
basolateral compartment of each well and 100 µl HBSSCM (without lucifer yellow) was added to the apical chamber. After incubating the cells for one hour at 37°C/95% humidity/5%CO₂ the receiver (apical) chamber and the donor chamber (basolateral) was sampled and the lucifer yellow-related fluorescence determined using a BioTEK SynergyMX plate reader (Winooski, VT). Wells were rejected if the permeability of the lucifer yellow was above 2%.

Apparent permeability ($P_{app}$) for both the MDCK wild type and the MDR1 transfected MDCK cells were calculated using eq.1, where ($V_r$) is the volume of buffer in the receiver, (A) is the surface area of the Transwell membrane (0.14 cm$^2$), (t) is the incubation time in seconds and drug (r) and (d) are the drug concentrations of the donor and receiver wells respectively at time t.

$$P_{app} = \frac{V_r}{(A\cdot t) \cdot \frac{[\text{Drug}]_r}{[\text{Drug}]_d}} \tag{1}$$

The directional ratio for each cell line was calculated by dividing the apparent permeability value for the basolateral-to-apical direction by the apparent permeability value for the apical-to-basolateral direction as eq. 2.

$$\text{efflux ratio} = \frac{P_{appB\rightarrow A}}{P_{appA\rightarrow B}} \tag{2}$$

The net efflux ratio was calculated by the dividing the directional ratio value for the MDR1 transfected cell line by the directional ratio of the untransfected MDCK cells using eq.3. A net efflux ratio above 2.0 was the criterion used to define significant substrate-transporter interaction (Polli et al., 2001).

$$\text{Net efflux ratio} = \frac{\text{efflux ratio}_{MDR1}}{\text{efflux ratio}_{wt}} \tag{3}$$

The mean and standard deviation was calculated from three wells for each direction within the same experiment.

**Animals.** Male CF-1 mdr1a(+/+) and mdr1a(-/-) mice (29-35 g) were purchased from Charles River Laboratories (Wilmington, MA). All mice were group housed on a 12:12-h
light/dark schedule with ad libitum access to food and water. Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Washington University School of Medicine (St. Louis, MO).

Previous studies in our laboratory showed that a 1 mg/kg dose of norbuprenorphine in wild type mice elicited a significant decrease in respiratory rate (Brown et al., 2011). Based on the \textit{in vitro} indication that norbuprenorphine is a P-gp substrate and the hypothesis that decreased efflux from the brain in a P-gp deficient mouse may result in increased norbuprenorphine effects, a pilot study was performed to determine the norbuprenorphine dose that would elicit the same respiratory effect in the \textit{mdr1a}(+/+) mice as the 1 mg/kg dose administered to the \textit{mdr1a}(+/-) mice. \textit{mdr1a}(+/+) and \textit{mdr1a}(+/-) mice (n=4 each) were used in the pilot study. A 0.25 mg/kg dose resulted in a 60% decrease in respiratory rate in the \textit{mdr1a}(+/-) mice, which was the same response seen in the \textit{mdr1a}(+/+) mice administered 1mg/kg. For all further experiments, these equieffective doses of norbuprenorphine were used.

\textbf{Collection of tissue samples.} P-gp competent \textit{mdr1a}(+/+) and P-gp deficient \textit{mdr1a}(+/-) mice (n=22 each) were administered norbuprenorphine subcutaneously (1 mg/kg for \textit{mdr1a}(+/+) and 0.25 mg/kg for \textit{mdr1a}(+/-) mice). At 5, 10, 20, 30, 45, and 60 minutes post-dose, respiratory rate and antinociception were assessed. Mice were anesthetized with sevoflurane and blood collected by cardiac puncture into heparinized Microtainers (BD Biosciences, Franklin Lakes, NL). Blood was centrifuged at 14,000 rpm for 1 minute to separate plasma. After exsanguination, whole brains were harvested and flash frozen. Plasma and brain were stored at -20°C until analysis.

\textbf{Quantitation of norbuprenorphine, norbuprenorphine-3-glucuronide, buprenorphine and buprenorphine-3-glucuronide.} Brain and plasma were prepared for analysis by LC-MSMS as previously described (Brown et al., 2011). In vitro samples were
prepared by the addition of d₅-norfentanyl (100 ng/ml) as an internal standard. LC-MSMS was used to quantify norbuprenorphine and norbuprenorphine-3-glucuronide in brain and plasma, and all four compounds in the in vitro assay samples. LC-MSMS as analysis was performed as previously described (Brown et al., 2011). Brain concentrations were corrected for drug present in brain vasculature, assuming 1% of total brain weight due to brain vasculature. No correction was applied to plasma drug concentrations.

**Tail-flick antinociception assay.** A tail flick assay (Montana et al., 2009) was used to test the antinociceptive effect of norbuprenorphine in mdr1(-/-) and mdr1(+/+) mice. Tail flick latency, defined by the time in seconds for tail withdrawal from a warm water bath (52°C) was measured as previously described (Brown et al., 2011), using an IITC 500 warm water tail immersion test analgesia meter (IITC Life Science, Woodland Hills, CA). Baseline tail flick latency was determined for each mouse prior to drug dosing. A cut-off of 10 seconds was used to prevent tissue damage. Animals not responding within 3 seconds were excluded from the assay. Maximum Possible Effect (MPE) was calculated as: \([\frac{(T_1-T_0)}{(T_2-T_0)}] \times 100\), where \(T_0\) and \(T_1\) represent latencies before and after drug administration, and \(T_2\) is the cut-off time. Tail flick latency was obtained for each animal after removal from a plethysmograph chamber and record of its respiratory rate.

**Unrestrained whole body plethysmography.** Measurements of respiratory rate were obtained using unrestrained whole body plethysmography (Buxco Research Systems, Wilmington, NC), as previously described (Brown et al., 2011). The plethysmograph consisted of eight animal chambers with orifices for entry and exit of breathing air, and a 1-mL syringe permitting calibrations, connected to a differential pressure transducer. The air entry orifice was connected to a source of compressed breathing air. Immediately prior to each experiment, each chamber was calibrated with 1 ml of room air. Each awake mouse was placed in a chamber immediately following administration of norbuprenorphine. Respiratory parameters were
recorded for 5, 10, 20, 30, 45, and 60 minutes. Respiratory values were converted by Biosystems XA software (Buxco Research Systems).

**Statistical Analysis.** Results are expressed as the mean ± SD. Two-way repeated measures analysis of variance (time versus group), followed by the Student-Newman-Keuls test, was used to test for significant differences between groups in mouse experiments (Sigma Plot 12.2, Systat Software, San Jose, CA).

Norbuprenorphine concentration-effect data in mdr1a(-/-) and mdr1a(+/-) mice were analyzed by non-linear regression fitting (Sigma Plot 12.2) of the sigmoid Emax model (eq. 4) to antinociception versus plasma or brain concentrations, where Emax was defined as 100%, and gamma was constrained to be the same for mdr1a(-/-) and mdr1a(+/-) mice, as described previously (Kalvass et al., 2007).

\[
\%MPE = \frac{E_{\text{max}} \cdot C}{E_{C_{50}} + C} 
\]  

(eq. 4)
RESULTS

In vitro transport results. The well-established transwell assay using MDCK cells transfected with human MDR1 was used to determine whether buprenorphine, norbuprenorphine or their glucuronide metabolites were substrates for P-gp. Transport activity for each drug was measured in the basolateral-to-apical (B→A) and the apical-to-basolateral (A→B) direction across MDCK parental and MDR1 transfected cell lines (table 1). In the MDR1 transfected MDCK cells the B→A transport of norbuprenorphine was approximately 20-fold greater than that observed for apical-to-basolateral transport (table 1). When the ratio was corrected for the influence of the endogenous transporters (by comparison against non-transfected cells) the net efflux ratio was 9. Buprenorphine, buprenorphine-3-β-D-glucuronide, and norbuprenorphine-3-β-D-glucuronide all had net efflux ratios less than the cutoff of 2.0 used to define a transporter substrate (table 1). Loperamide, a known P-gp substrate (Polli et al., 2001), was used as a positive control. The net efflux ratio for loperamide was 13.

Disposition of norbuprenorphine in mdr1a(-/-) and mdr1a(+/+) mice. The brain to plasma ratio of norbuprenorphine in the mdr1a(+/+) mice was low (<0.1) at the 5 minute time point, and remained essentially unchanged throughout the experiment (fig 1). In contrast, the brain to plasma ratio in the mdr1a(-/-) mice was >0.1 at the 5 minute time point, and continued to increase over the time course of the experiment, approaching 1 by the 60 minute time point (fig 1). For norbuprenorphine 3-glucuronide there was no significant difference in the brain to plasma ratios in the mdr1a(-/-) and mdr1a(+/+) mice, and the ratio was less than 0.1 throughout the experiment.

The plasma concentrations of norbuprenorphine were 4-fold less in the mdr1a(-/-) mice than the mdr1a(+/+) mice, consistent with the mdr1a(-/-) mice receiving one fourth the dose of that in the mdr1a(+/+) mice (fig 1). Plasma concentrations of norbuprenorphine-3-glucuronide were also 4-fold less in the mdr1a(-/-) mice than the mdr1a(+/+) mice, again consistent with the
norbuprenorphine dose difference. Plasma norbuprenorphine-3-glucuronide: norbuprenorphine concentration ratios were similar in the mdr1a(-/-) and mdr1a(+/+) mice.

**Antinociceptive effect of norbuprenorphine in mdr1a(-/-) and mdr1a(+/+) mice.** In the mdr1a(+/+) mice, norbuprenorphine peak effect was 30% of Maximum Possible Effect (MPE) (fig 2). Maximal antinociception occurred 20 minutes post-dosing, and effects returned to baseline at 45 minutes. In contrast, the mdr1a(-/-) mice had markedly increased magnitude and duration of norbuprenorphine antinociception. The effect peaked at 100% MPE at 20 minutes, and only declined to 80% by 60 minutes (fig 2).

Norbuprenorphine concentration-effect curves were constructed for both plasma and brain concentrations. There was a substantial leftward shift of the plasma concentration-effect curve in the mdr(-/-) compared with the wild-type mice (fig 3). The norbuprenorphine plasma EC$_{50}$ was 14 ± 5 in the mdr1a(-/-) compared to 471 ± 255 in the mdr1(+/+) mice. In contrast, there was no apparent difference between the two mouse strains in the brain concentration-effect relationship for buprenorphine, which could be described by a common exponential, with the brain EC$_{50}$ of 6 ± 1 (fig 4).

**Norbuprenorphine respiratory effects in mdr1a(-/-) and mdr1a(+/+) mice.** By experimental design (see Methods) a dose of 0.25 mg/kg norbuprenorphine elicited the same effect in mdr1a(-/-) mice as was seen in the mdr1a(+/+) mice which received 1 mg/kg (fig 6). Respiratory rates decreased approximately 60% from baseline in both groups. There was no change in tidal volume following administration of norbuprenorphine in either mdr1a(-/-) or mdr1a(+/+) mice.

**Behavioral Observations.** During the course of the experiment, several behaviors were observed in the P-gp deficient mdr1a(-/-) mice that received norbuprenorphine. At 15 minutes after administration of norbuprenorphine, the mdr1a(-/-) mice exhibited Straub tails (Bibbey et al., 1960). The mdr1a(-/-) mice exhibited loss of the righting reflex (Oka et al., 1992; Kamei et al.,
1996), with the mice unable to right themselves upon being released after the tail flick procedure. In comparison, the *mdr1a(+/+)* did not show Straub tails or loss of righting reflex. Both groups of *mdr1a(+/+)* and (-/-) mice appeared equally sedated after norbuprenorphine, with minimal movements approximately 15 minutes after norbuprenorphine dosing.
DISCUSSION

The purpose of this investigation was to evaluate P-gp-mediated transport of buprenorphine, norbuprenorphine, norbuprenorphine-3-glucuronide and buprenorphine-3-glucuronide in vitro, and test the hypothesis that P-gp mediated efflux limits brain access of norbuprenorphine (and possibly norbuprenorphine-3-glucuronide) and influences norbuprenorphine antinociception. P-gp transfected MDCK cells were used to screen for P-gp substrates specifically, rather than as a model for blood-brain transport more generally. The major result was that norbuprenorphine was a P-gp substrate both in vitro and in vivo. As measured by the net efflux ratio in transfected cells, P-gp-mediated norbuprenorphine efflux was substantial, and approximated that of the unambiguous P-gp substrate loperamide (Polli et al., 2001) (9 vs 13, respectively). P-gp-mediated norbuprenorphine brain efflux was also observed in vivo. In mdr1a(+/+) animals, the norbuprenorphine brain/plasma ratio was negligible and constant. In contrast, in mdr1a(-/-) animals, the norbuprenorphine brain/plasma ratio increased steadily, approaching unity. This demonstrates that P-gp represents a major barrier to norbuprenorphine entering the brain and that elimination of transporter function resulted in increased norbuprenorphine brain concentrations, which essentially equaled plasma concentrations. Thus, absent P-gp-mediated efflux, norbuprenorphine accumulates in the brain until the drug is equally distributed between brain and plasma. The low norbuprenorphine brain concentration in mdr1a(+/+) mice is likely due to a combination of low passive permeability and substantial P-gp-mediated efflux (Ohtani et al., 1995).

P-gp-mediated brain access significantly influenced the pharmacologic effect of norbuprenorphine. Antinociception was greater in both magnitude and duration in mdr1a(-/-) compared to mdr1a(+/+) mice despite the mdr1a(-/-) mice receiving one-fourth the norbuprenorphine dose of the mdr1a(+/+) mice. Moreover, the duration of norbuprenorphine antinociception in mdr1a(-/-) mice was even greater than that observed after a maximally
antinociceptive buprenorphine dose in \textit{mdr1a}(+/+) mice (Brown et al., 2011). There was a substantial shift of the norbuprenorphine plasma concentration-effect curve for antinociception, and more than an order of magnitude difference in the plasma EC\textsubscript{50} between \textit{mdr1a}(−/−) and \textit{mdr1a}(+/+) mice, yet no difference in the brain concentration-effect curves. In addition, some characteristic opioid-induced behaviors were observed after norbuprenorphine in \textit{mdr1a}(−/−) but not \textit{mdr1a}(+/+) mice. The \textit{mdr1a}(−/−) mice administered norbuprenorphine exhibited Straub tails - the tail held erect due to opioid-induced contraction of the sacrococcygeus muscle (Bilbey et al., 1960; Aceto et al., 1969). This has been shown to be mediated through the central \(\mu_2\)-opioid receptor subtype (Nath et al., 1994). The \textit{mdr1a}(−/−) mice also exhibited loss of righting reflex, another \(\mu_2\)-opioid receptor mediated effect (Oka et al., 1992; Kamei et al., 1996). Together, all these findings strongly support the hypothesis that P-gp regulates brain access of norbuprenorphine, and that greater norbuprenorphine brain access in P-gp-deficient mice caused greater antinociceptive and behavioral effects.

Norbuprenorphine is a mu, delta, and kappa receptor agonist, as shown \textit{in vitro} (Huang et al., 2001; Brown et al., 2011). Thus, the poor antinociceptive effects of norbuprenorphine in wild-type mice could be due to limited brain exposure rather than low intrinsic analgesic efficacy of norbuprenorphine. While norbuprenorphine was shown to have an analgesic effect one-fourth that of buprenorphine following an intraventricular dose in rats (Ohtani et al., 1995), intraventricular dosing in a wild type animal may still be accompanied by substantial P-gp mediated efflux and therefore less overall brain exposure.

Both buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide are abundant buprenorphine metabolites in humans \textit{in vivo} (McCance-Katz et al., 2006; McCance-Katz et al., 2007) and were found to be biologically active in mice (Brown et al., 2011). The potential contribution of norbuprenorphine-3-glucuronide to norbuprenorphine effects and influence of P-gp deficiency on norbuprenorphine effects were unknown. Therefore P-gp-mediated
norbuprenorphine-3-glucuronide transport was evaluated, along with that of buprenorphine and buprenorphine-3-glucuronide. In vitro, neither buprenorphine, buprenorphine-3-glucuronide, nor norbuprenorphine-3-glucuronide were P-gp substrates. The net efflux ratio was approximately 1 for each. In vivo, norbuprenorphine-3-glucuronide brain/plasma concentration ratios after norbuprenorphine were very small, and unaffected by P-gp deficiency, indicating that norbuprenorphine-3-glucuronide has limited brain access, and P-gp does not influence norbuprenorphine-3-glucuronide efflux or brain concentrations in vivo.

Although brain norbuprenorphine-3-glucuronide concentrations were low, they were not zero, indicating that despite the highly hydrophilic nature of this glucuronide, it did pass the blood-brain barrier. Limited but detectable brain access of norbuprenorphine-3-glucuronide is consistent with previous results, which found norbuprenorphine-3-glucuronide in brain after administration of either norbuprenorphine or the glucuronide, and a small pharmacologic effect of norbuprenorphine-3-glucuronide (Brown et al., 2011). The determinants of norbuprenorphine-3-glucuronide brain access, including passive permeability and/or active uptake, are not known. Organic anion transporting polypeptides are expressed in both human and murine brain microvessel endothelial cells (Hagenbuch and Meier, 2004). These transporters can transport glucuronide conjugates of drugs (Hagenbuch and Meier, 2004) and may be responsible, in vivo, for brain exposure of glucuronides. Further testing is required to determine any possible role for uptake transporters in the brain exposure of norbuprenorphine-3-glucuronide (or buprenorphine-3-glucuronide).

The norbuprenorphine-3-glucuronide results provide additional insights into the mechanism of norbuprenorphine effects. Norbuprenorphine-3-glucuronide was shown previously to have high affinity for kappa and nociceptin but not mu or delta receptors, and, when administered to mice, substantially decreased tidal volume but not respiratory rate (Brown et al., 2011). In the present experiment, brain norbuprenorphine-3-glucuronide concentrations
were very low after norbuprenorphine, and did not differ between $mdr1a(+/+) \text{ and } mdr1a(-/-)$ mice. Respiratory rate but not tidal volume was affected in both $mdr1a(+/+) \text{ and } mdr1a(-/-)$ mice. This suggests that norbuprenorphine-3-glucuronide did not contribute to the respiratory effect following a norbuprenorphine dose. Sedation is another effect of both norbuprenorphine and norbuprenorphine-3-glucuronide (Brown et al., 2011). Both the $mdr1a(-/-) \text{ and } mdr1a(+/+) \text{ mice were equally sedated following norbuprenorphine, despite the large differences in brain concentrations of both norbuprenorphine and norbuprenorphine-3-glucuronide between the two strains. Thus, it cannot be concluded whether the sedation observed in this study was attributable to norbuprenorphine, norbuprenorphine-3-glucuronide, or both.}

The role of brain access via passive permeability and/or P-gp mediated efflux and contribution to the respiratory effect of norbuprenorphine is not yet clear. The dose of norbuprenorphine given to the $mdr1a(-/-)$ mice was selected to match the respiratory effects of norbuprenorphine in the $mdr1a(+/+) \text{ mice. Interestingly, the significant respiratory depression in } mdr1a(+/+) \text{ mice occurred despite very low brain norbuprenorphine concentrations. Additionally, the time course of decreased respiratory rate did not parallel the time course of norbuprenorphine brain concentration. While there was an increase in brain norbuprenorphine corresponding to the decrease in respiratory rate in the $mdr1a(-/-)$ mice, there was no change in brain norbuprenorphine in the $mdr1a(+/+) \text{ mice. A plausible hypothesis for the profound norbuprenorphine effect despite such low brain concentrations is high respiratory depressant potency. While norbuprenorphine potency has been modeled in silico (Yassen et al., 2007), potency of norbuprenorphine respiratory depression remains to be elucidated in vivo. Further, the receptor type involved in mediating norbuprenorphine-induced respiratory depression is still unresolved.}

P-gp magnifies the discrepancy between pharmacologic effects of buprenorphine and norbuprenorphine. Buprenorphine is not a P-gp substrate (Nekhayeva et al., 2006; Hassan et
al., 2009; Tournier et al., 2010) and P-gp does not influence the pharmacologic effect of buprenorphine in mice (Hassan et al., 2009). In contrast, norbuprenorphine clearly is a P-gp substrate. Norbuprenorphine has significant respiratory effects in both mdr1a(-/-) and mdr1a(+/+) mice, but only in the absence of P-gp does norbuprenorphine have a substantial antinociceptive effect. This suggests that the sites or mechanism of action for norbuprenorphine analgesia and respiratory depression may be distinct, and that P-gp mediated efflux restricts norbuprenorphine access to the analgesia effect site but not the respiratory effect site. This could be due to differential expression of P-gp in various locations of the blood-central nervous system brain barrier. For example, there is lower P-gp expression in murine endothelial cells of the blood-spinal cord barrier compared with the blood-brain barrier (Ge and Pachter, 2006). Additionally, P-gp influenced blood-brain but not blood-spinal cord morphine distribution in mdr1a(-/-) compared to mdr1a(+/+) mice (Zong and Pollack, 2000). Thus differences in brain P-gp and norbuprenorphine efflux between mdr1a(+/+) and mdr1a(-/-) mice may be greater than differences in spinal cord P-gp and efflux, potentially influencing analgesia more than respiratory depression, depending on their respective sites of action.

The influence of P-gp on norbuprenorphine effects can be compared to that of other opioids. The increase in maximum antinociception (MPE) in mdr1a(-/-) compared to mdr1a(+/+) mice was 60% for norbuprenorphine, but 20, 40, and 50% for methadone (Thompson et al., 2000; Hassan et al., 2009), morphine (Zong and Pollack, 2000), and fentanyl (Hamabe et al., 2006), respectively. Also, the eight-fold increase in the brain/plasma norbuprenorphine concentration ratio in the absence of P-gp mediated efflux is greater than that of other opioids. Brain/plasma concentration ratios of morphine, alfentanil, and methadone only increased 2-, 2.4, and 3.5-fold, respectively, in mdr1(-/-) compared to mdr1(+/+) mice (Zong and Pollack, 2000; Kalvass et al., 2007; Hassan et al., 2009). Moreover, the more than order of magnitude decrease in plasma EC50 in the absence of P-gp was much greater for norbuprenorphine than
for other opioids, such as alfentanil (Kalvass et al., 2007). Thus the degree to which P-gp impedes brain access and antinociception is greater for norbuprenorphine than these other opioids.

The role of P-gp in mediating brain access of norbuprenorphine in humans, and the pharmacologic contribution of norbuprenorphine to buprenorphine clinical effects, are unknown. If, however, these are clinically relevant, then findings described in this report may explain, in part, recent clinical observations about buprenorphine (Bruce and Altice, 2006; McCance-Katz et al., 2007). Atazanavir and atazanavir/ritonavir increased plasma buprenorphine and norbuprenorphine concentrations 1.3-2.5 fold, albeit not into ranges shown previously to cause side effects (McCance-Katz et al., 2007). Nevertheless, the interaction caused some subjects became sedated. The adverse side effects after buprenorphine/atazanavir were attributed to inhibition of CYP3A4-mediated buprenorphine metabolism by atazanavir. However atazanavir is also a P-gp inhibitor (Bierman et al., 2010), and, in light of the findings in the present report, it is possible that atazanavir-enhanced buprenorphine effects could be attributable, at least in part, to P-gp inhibition and increased norbuprenorphine brain access. Tipranavir/ritonavir had no effect on plasma buprenorphine concentrations but diminished norbuprenorphine concentrations to one-fifth of control, and had no effect on opioid withdrawal (Bruce et al., 2009). While this was considered mechanistically unclear, tipranavir and ritonavir are both P-gp inhibitors (Storch et al., 2007), and might have therefore increased brain norbuprenorphine exposure and contributed to obviating withdrawal. Rifampin induced buprenorphine clearance and caused significant withdrawal in opioid-dependent patients (McCance-Katz et al., 2011). While withdrawal was attributed to diminished buprenorphine concentrations, the decrease in plasma norbuprenorphine concentrations was substantially greater than that of buprenorphine (8- vs 3-fold). Since rifampin induces brain P-gp (Bauer et al., 2006), the combined effects of rifampin to decrease plasma norbuprenorphine concentrations and upregulate norbuprenorphine brain...
efflux, could have markedly decreased brain norbuprenorphine exposure, and contributed to the withdrawal. Nevertheless, the role of norbuprenorphine and P-gp-mediated brain access in humans remains unknown.

Two limitations of the present investigation merit mention. Buprenorphine was found to bind readily to the polystyrene used for the *in vitro* transwell studies, which potentially confounded the accurate determination of the extent of transcellular movement. Due to differences in the surface areas of the apical and basolateral compartments, correction for nonspecific binding was not possible. Therefore the buprenorphine data should be interpreted carefully. Nonspecific buprenorphine binding has also been reported previously (Tournier et al., 2010). Due to the unexpectedly large effect of P-gp on brain norbuprenorphine access, there was not sufficient overlap of norbuprenorphine brain concentrations between *mdr1a(−/−)* and *mdr1a(+/+) mice to separately model the brain concentration-antinociception data in the two groups, thus they were modeled together.

In conclusion, the limited antinociceptive effect of norbuprenorphine is due to P-gp mediated inhibition of brain access. P-gp does not influence brain disposition of buprenorphine-3-glucuronide or norbuprenorphine-3-glucuronide. These findings add to the complexity of buprenorphine pharmacology.
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AUTHOR CONTRIBUTIONS:

Participated in research design: Brown, Campbell, Kharasch

Conducted Experiments: Brown, Campbell, Crafford, Regina

Contributed new reagents or analytic tools: Holtzman

Performed data analysis: Brown, Campbell, Crafford

Wrote or contributed to the writing of the manuscript: Brown, Campbell, Kharasch
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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Disposition of norbuprenorphine and norbuprenorphine glucuronide. Concentrations of norbuprenorphine (A,B) and norbuprenorphine-3-glucuronide (C,D) were determined in brain homogenate (circles) and plasma (triangles) were determined by LC-MS/MS. Results are shown for mdr1a(+/+) (open symbols) and mdr1a(-/-) (closed symbols) mice. Measured concentrations are shown in panels A and C; norbuprenorphine and norbuprenorphine glucuronide brain/plasma ratios are shown in panels B and D. Each data point is the mean ± SD (n=4). *Significantly different from mdr1a(+/+) controls (p<0.05) by two-way repeated-measures analysis of variance.

Figure 2. Antinociceptive effects of norbuprenorphine in mdr1a(-/-) and wild type mice. Time to withdrawal of the tail from a hot water (52°C) bath, or tail flick latency (TFL) was measured after subcutaneous injection of vehicle or norbuprenorphine (1 mg/kg in the mdr1a(+/+) mice; 0.25 mg/kg in the mdr1a(-/-) mice). The % maximum possible effect (MPE) of the mdr1a(+/+) mice (A) and the mdr1a(-/-) mice (B) was calculated every 15 min for 60 min. Each data point is the mean ± SD (10/group). *Significantly different from vehicle control (p<0.05) by two-way repeated-measures analysis of variance.

Figure 3. Relationship between antinociception and plasma norbuprenorphine concentration. The relationship shown is following subcutaneous injections of 0.25 mg/kg norbuprenorphine in mdr1a(-/-) mice (closed circles) and 1.0 mg/kg in mdr1(+/+) mice (open circles). Data are mean ± SD (n=4). Lines represent the fit of a sigmoidal Emax model to the effect data.

Figure 4. Relationship between antinociception and brain norbuprenorphine concentration. The relationship shown is following subcutaneous injections of 0.25 mg/kg
norbuprenorphine in mdr1a(-/-) mice (closed circles) and 1.0 mg/kg in mdr1(+/+) mice (open circles). Data are mean ± SD (n=4). Lines represent the fit of a sigmoidal Emax model to the effect data.

Figure 5. Respiratory rate in mdr1a(-/-) and wild type mice administered norbuprenorphine. Respiratory rate and tidal volume in mdr1a(+/+) (A) and mdr1a(-/-) (B) mice following subcutaneous injection of norbuprenorphine or saline vehicle were measured by unrestrained whole body plethysmography. Results are mean ± SD (4 per group). *Significantly different from vehicle control (p<0.05) by two-way repeated-measures analysis of variance. There was no effect of norbuprenorphine on tidal volume.
**Table 1. Bidirectional transport across untransfected and MDR1 transfected MDCK cells.**

Data are presented as the apparent permeability ($P_{app}$). The efflux ratio for each cell line was determined as $P_{app} (B \rightarrow A)/P_{app} (A \rightarrow B)$. The net efflux ratio was calculated as Efflux ratio$_{MDR1}$/Efflux ratio$_{MDCK}$. Compounds are considered P-gp substrates if the net efflux ratio is greater than 2.0, as defined in the Methods. Results are the mean ± standard deviation for triplicate wells performed on the same day.

|                      | $P_{app}$ (nm/sec) |  |  |  |  |  |  |  |
|----------------------|-------------------|---|---|---|---|---|---|
|                      | B→A/ A→B          | MDCK | MDR1 | MDCK | MDR1 | Net Efflux |
| **buprenorphine**    |                   |      |      |      |      |            |
| B→A                  | 793±26            | 821±153 | 81.7 | 45.5 | 0.6      |
| A→B                  | 10±3              | 18±4    |      |      |          |
| **norbuprenorphine** |                   |      |      |      |      |            |
| B→A                  | 211±5             | 170±6   | 2.2  | 19.8 | 9         |
| A→B                  | 91±5              | 8±1     |      |      |          |
| **buprenorphine 3-glucuronide** |       |      |      |      |      |            |
| B→A                  | 166±1             | 156±19  | 3.8  | 3.8  | 1         |
| A→B                  | 44±10             | 41±11   |      |      |          |
| **norbuprenorphine 3-glucuronide** |         |      |      |      |      |            |
| B→A                  | 159±37            | 222±49  | 1.1  | 0.9  | 0.9       |
| A→B                  | 197±12            | 205±19  |      |      |          |
| **Loperamide**       |                   |      |      |      |      |            |
| B→A                  | 144±17            | 117±43  | 1.8  | 23.3 | 13        |
| A→B                  | 80±13             | 3±1     |      |      |          |
Figure 1.
Figure 2.
Figure 3.

The graph illustrates the plasma concentration (ng/mL) on the x-axis ranging from 10 to 1000, and % MPE on the y-axis ranging from -20 to 100. The graph compares mdr (+/+) and mdr (-/-) genotypes. The data points are accompanied by error bars, indicating variability in the measurements.
Figure 4.

![Brain concentration (ng/mL) vs. % MPE for mdr1(+/+) and mdr1(-/-) mice.](image-url)
Figure 5.

A and B: Graphs showing the change in breaths per minute over time with and without norbuprenorphine.