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Opiates inhibit paclitaxel uptake by P-glycoprotein in preparations of human placental inside-out vesicles

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ABSTRACT

The use of either methadone or buprenorphine for treatment of the pregnant opiate-dependent patient improves maternal and neonatal outcome. However, patient outcomes are often complicated by neonatal abstinence syndrome (NAS). The incidence and severity of NAS should depend on opiate concentration in the fetal circulation. Efflux transporters expressed in human placental brush border membranes decrease fetal exposure to medications by their extrusion to the maternal circulation. Accordingly, the concentration of either methadone or buprenorphine in the fetal circulation is, in part, dependent on the activity of the efflux transporters. The objective of this study was to characterize the activity of P-gp and its interaction with opiates in the placental apical membrane. Therefore, brush border membrane vesicles were prepared from human placenta. The vesicles were oriented approximately 75% inside-out, exhibited saturable ATP-dependent uptake of P-gp substrate [^{3}H]-paclitaxel with an apparent K_t of 66 ± 38 nM and V_{max} of 20 ± 3 pmol mg protein^{-1} min^{-1}. Methadone, buprenorphine, and morphine inhibited paclitaxel transport with apparent K_i of 18, 44, and 90 μM, respectively. Our data indicate that a method has been established to determine the activity of the efflux transporter P-gp, expressed in placental brush border membranes, and the kinetics for the transfer of its prototypic substrate paclitaxel. Furthermore, the method was used to determine the effects of methadone, buprenorphine, and morphine on paclitaxel transfer by placental P-gp and revealed that they have higher affinity to the transporter than its classical inhibitor verapamil (K_i, 300 μM).

1. Introduction

One of the long term goals of our laboratory is to determine the role of human placenta in the biodisposition of pharmacotherapeutic agents used for the treatment of the pregnant opiate addicts. Methadone is considered the gold standard for pharmacotherapy of the pregnant opiate-dependent patients [1]. Methadone maintenance programs improve maternal and neonatal outcome; although they could be associated with neonatal abstinence syndrome (NAS). However, the incidence and severity of NAS do not correlate with the administered dose of methadone [2]. Buprenorphine, a partial μ-opioid agonist, is also used for the treatment of the opiate-dependent adults. Furthermore, neonates born to buprenorphine-treated patients exhibited milder NAS and shorter duration of hospitalization than those treated with methadone [3–5]. There are conflicting reports on whether the incidence and severity of NAS correlates with the regimen, including dose, of methadone or buprenorphine. This led us to postulate that the incidence and severity of NAS should correlate with the concentration of the opiate in the fetal, rather than maternal, circulation. Accordingly, our working hypothesis is that human placenta acts as a functional barrier which decreases fetal exposure to medications/xenobiotics. The functions of the placental barrier include regulation of transplacental transfer of a drug, its biotransformation by trophoblast tissue metabolic enzymes, and its extrusion from the feto-placental unit by efflux transporters.

The transplacental transfer of methadone and buprenorphine was previously investigated in our laboratory using the technique of dual perfusion of human placental lobule. The data obtained revealed that the rate of methadone transfer from the maternal to fetal circuit was higher (29.4 ± 4.6%) than that for buprenorphine

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(11.6 ± 2.5%) [6,7]. However, the ratio of the concentration of buprenorphine retained in the placental tissue to that in the maternal or fetal circuits was significantly greater than for methadone. This data indicates that placental distribution, i.e., extent of transfer and retention/accumulation in the tissue, is different between the two opiates.

Placental distribution of a drug depends on many factors including: its physicochemical properties (charge, molecular weight, protein binding); physiological properties of the placenta (blood flow, gestational age); the expression and activity of metabolizing enzymes and efflux transporters. In recent years, a large number of transporters were identified in human placental syncytiotrophoblast tissue [8,9]. In general, efflux transporters are localized in the apical membrane of syncytiotrophoblast tissue and extrude their substrates (endogenous compounds or certain drugs) from the feto-placental unit to the maternal circulation thus decreasing fetal exposure. The activity of the ATP Binding Cassette (ABC) family of efflux transporters appears to have a major role in protecting the fetus from exposure to xenobiotics and endogenous metabolites in the maternal circulation.

One of the most highly characterized placental ABC transporters is P-glycoprotein (P-gp). P-gp effluxes a wide variety of compounds in several tissues, namely the blood–brain barrier, intestinal brush border membranes, and placental apical membranes [10,11]. Previous investigations in our laboratory demonstrated that buprenorphine, methadone, and morphine interact/bind to P-gp, as determined by the stimulation of ATP hydrolysis in human cDNA-P-gp membrane preparations [12]. The role of placental P-gp was confirmed using the *ex vivo* dual perfusion of placental lobule (DPPL). Data obtained revealed that the addition of a P-gp inhibitor to the perfusion medium increased the concentration of methadone, but not buprenorphine, in the fetal circuit [12,13]. Therefore, it appears that methadone and buprenorphine interact with P-gp (as judged by stimulation of ATP hydrolysis) and methadone — but not buprenorphine — is extruded by P-gp from the feto-placental unit. However, a direct determination of the activity of placental P-gp and the kinetics of its transfer of these potential substrates remains unanswered.

Placental membrane vesicles allow the direct determination of the transport kinetics of potential P-gp substrates. P-gp is localized in the brush border membrane of placental syncytiotrophoblast, a polarized epithelium expressing different transporters/proteins on the brush border (apical) and basal membranes. The asymmetry of the syncytiotrophoblast membrane allows the separation and isolation of the brush border membranes. Vesicles formed during the preparation of brush border membranes assume two configurations: inside-out (IOV) and right-side out (ROV). IOV preparations containing P-gp have the cytoplasmic ATP-binding domain and the substrate binding site on the outside of the plasma membrane bilayer. Therefore, the uptake of a compound by the IOVs determines the activity of the transporter in its transfer from the outside to the inside of the vesicle. Consequently, a preparation enriched in IOVs can be used to investigate the transport of radiolabeled substrate of P-gp which, following active transport, becomes 'trapped' inside the vesicle. The IOV preparation can be utilized to determine the *in vitro* time-dependent, P-gp-mediated accumulation of a radioisotope of the substrate, as well the kinetics of transport (K_t and V_{max}).

Therefore, the goals of this investigation were: (1) characterize the kinetics of placental P-gp-mediated transport of its prototypic substrate, paclitaxel, using placental apical membrane inside-out vesicles; (2) correlate the activity of P-gp-mediated transport of a substrate with its protein expression in individual placentas; (3) determine the effects of the opiates methadone, buprenorphine, and morphine on placental P-gp transport of its prototypic substrate paclitaxel. Information on the activity of P-gp interaction with opiates used as medications during pregnancy would lead to a better understanding of the mechanisms underlying the extent of fetal exposure to these medications, and consequently the incidence and severity of NAS.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise mentioned. The opiates buprenorphine, morphine, and methadone and their tritiated isotopes were a gift from the National Institute on Drug Abuse drug supply unit. Paclitaxel and paclitaxel [*o*-benzamido-^{3}H] (38–Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). The murine monoclonal antibodies (mAb C219) were purchased from Signet Laboratories (Dedham, MA). Actin (C-2) mouse monoclonal antibodies and goat anti-mouse horseradish peroxidase-conjugated antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

2.2. Clinical material

A staff of trained research nurses is responsible for transporting the placentas immediately after delivery to our laboratory according to a protocol approved by the Institutional Review Board of UTMB. *Exclusion criteria*: documented drug abuse during pregnancy or infections with HIV or hepatitis. *Inclusion criteria*: term placentas (37–41 weeks) obtained from uncomplicated pregnancies or preterm deliveries (<37 weeks) of various etiologies.

2.3. Preparation of placental brush border membrane vesicles

Placental brush border membrane vesicles were prepared according to a previously reported method with modifications [14]. Tissue was dissected from the maternal side and rinsed twice in 0.9% NaCl. All of the following steps were carried out at 4 °C. The tissue was transferred to sucrose–HEPES-Tris (SHT) buffer (250 mM sucrose, 10 mM HEPES-Tris, pH 7.4), and stirred for 1 h to disrupt brush border membranes. The tissue lysate was filtered through two layers of woven cotton gauze, and the tissue was discarded. The filtrate was centrifuged at 800 × *g*: 10 min, to remove blood and cell debris. The supernatant was combined with 20 mM $MgCl_2$ (1:1 ratio) and stirred for 10 min. The solution was centrifuged at 10,500 × *g*, 10 min. The pellet was discarded and the supernatant was centrifuged at 20,000 × *g*, 20 min. The pellet, containing brush border membrane, was re-suspended in SHT buffer with a 26-gauge needle.

2.4. Electron microscopy

The pellet containing the brush border membrane vesicles was fixed in 2.5% formaldehyde and 0.03% $CaCl_2$. The pellet was then washed in 0.1 M cacodylate buffer and fixed in 1% OsO_4 in 0.1 M cacodylate buffer. The pellet was *en bloc* stained with 2% aqueous uranyl acetate, dehydrated in a graded series of ethanol, and embedded in Poly/Bed 812 (epoxy). Ultra thin sections were then cut on a Leica Ultracut S ultramicrotome, stained with lead citrate and examined in a Philips 201 electron microscope at 60 kV.

2.5. Alkaline phosphatase activity

The purity of brush border membrane preparation was assessed by determining the activity of alkaline phosphatase [15] using AnaSpec Sensolyte pNPP Alkaline Phosphatase Colorimetric Assay

Kit. Alkaline phosphatase from calf intestine was used as a standard.

2.6. Affinity chromatography

To maximize the proportion of IOVs, affinity chromatography was used to separate ROVs according to a previously reported method [16]. Briefly, lectin immobilized on an agarose column binds the N-acetyl-glucosaminyl residues of glycolipids and glycoproteins (extracellular) on the ROV, and retains the ROVs in the column. Methyl-α-D-mannopyranoside was used to displace the retained ROVs from the lectin affinity column following IOV elution. Vesicles in the inside-out configuration (IOVs) were used in all of the experiments sited in this report.

2.7. Acetylcholinesterase activity

To ratio of IOVs to the total present in the preparation is determined by the activity of acetylcholinesterase. Control vesicles were lysed in hypotonic buffer to represent 100% of acetylcholinesterase activity. Intact or lysed vesicles (5 mg/mL) were suspended in 0.1 M phosphate buffer containing 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) and 0.1 M acetylthiocholine chloride and incubated at room temperature for 3 min. Absorbance was read at 412 nm and the acetylcholinesterase activity was determined as moles of substrate hydrolyzed/min mg protein^{-1} using the formula: $R = ((1.22 \times 10^{-3}) \times \Delta A)/C$ (ΔA = change in absorbance, C = mg/mL protein). The percentage of inside-out vesicles was calculated as $R_{lysed} - R_{intact}$ = % inside-out (R_{lysed}: activity in lysed vesicles, R_{intact}: activity in intact vesicle preparation).

2.8. Determination of P-gp protein levels by Western blot

The brush–border membranes were prepared as described above. The total protein concentration in all samples was determined by detergent-compatible Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as a standard. Identification of P-gp expression was carried out using 7.5% SDS/polyacrylamide gel electrophoresis. The amount of sample protein loaded on each well was 10 μg. At the end of electrophoresis, the gel was electroblotted on nitrocellulose membranes overnight at 4 °C and a constant potential of 25 V. Blots were probed with primary murine monoclonal antibodies (mAb C219) diluted 1:200 and secondary goat anti-mouse horseradish peroxidase-conjugated antibodies diluted 1:1000. Detection of the protein bands was carried out by spot densitometry and digital imaging of the enhanced chemiluminescence spots. The amount of expressed β-actin was used to normalize the amount of P-gp in each loaded sample on the gel. A positive control consisted of human P-gp membranes (Gentest Corporation).

2.9. Uptake by membrane vesicles

P-gp transport activity was determined by the uptake of its radiolabeled prototypic substrate [^{3}H]-paclitaxel (38 Ci/mmole, 83 dpm/fmole), in placental IOVs according to a previously reported protocol [14]. Each reaction was carried out in SHT buffer (250 mM sucrose, 10 mM HEPES-Tris) containing 4 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg/mL creatine phosphokinase, either 2 mM ATP or 3 mM NaCl, and placental IOVs at a concentration of 0.05 μg/μL (7 μg total protein). The reaction was initiated by the addition of [^{3}H]-paclitaxel, at a final concentration ~70 nM unless otherwise indicated. The reaction was terminated after 1 min by the addition of 1 mL ice cold buffer, and vesicles were isolated using rapid filtration by a Brandel Cell Harvester. The amount of [^{3}H]-paclitaxel retained on the filter was determined by liquid scintillation analysis. Active transport was calculated as the difference in the amount of [^{3}H]-paclitaxel in the presence and absence of ATP and expressed as pmol mg protein^{-1} min^{-1}. P-gp-specific transport of paclitaxel was confirmed by the addition of monoclonal antibody C219 and the P-gp inhibitor, verapamil. P-gp interaction with the opiates was determined using varying concentrations of methadone, buprenorphine, or morphine as determined by their inhibition of [^{3}H]-paclitaxel uptake by placental IOVs.

2.10. Calculation of K_t and V_{max}

The uptake of a range of concentrations of [^{3}H]-paclitaxel by the membrane vesicles was determined. The apparent K_t and V_{max} were determined by the least-squares fit of the data obtained using the Michaelis–Menten equation. The reciprocal of [^{3}H]-paclitaxel uptake (1/uptake) was plotted vs. the reciprocal of [^{3}H]-paclitaxel concentration in the incubation medium ($1/c$) for each experiment. The K_t and V_{max} values for each experiment were obtained from the X- and Y-intercepts, respectively, and pooled together to calculate the mean ± SEM.

2.11. Calculation of K_i

The IC_{50} of four inhibitors (verapamil, methadone, buprenorphine, or morphine) was determined. The K_i was calculated using the apparent K_t for paclitaxel determined in our laboratory and the equation $K_i = (IC_{50})/(1 + [\text{paclitaxel}]/K_{t\ \text{paclitaxel}})$ [17].

3. Results

3.1. Electron microscopy of vesicle preparations

Fig. 1 shows an electron micrograph of human placental brush border membrane vesicles at low magnification (62,700×, scale bar 1 μm), and inset at high magnification (14,100×, scale bar 1 μm). Micrographs revealed single bilayer vesicles averaging 0.5–1 μm in diameter.

3.2. Vesicle composition and orientation

Alkaline phosphatase is an enzymatic marker for the brush border membrane of placental syncytiotrophoblast. The preparation of vesicles used was 12.4 ± 4-fold greater than syncytiotrophoblast cell lysate in its content of alkaline phosphatase, indicating that the procedure for preparing the vesicles enriched the purity of brush border membrane. Affinity chromatography was utilized to separate vesicles oriented inside-out. Acetylcholinesterase assay (n = 20) and ATP-dependent uptake of [^{3}H]-paclitaxel (n = 32) demonstrated 1.9 ± 0.2- and 2.5 ± 1.2-fold enrichment, respectively, in the proportion of IOVs. This data indicated that the affinity chromatography column doubled the proportion of IOVs in the preparations used in this investigation.

3.3. P-glycoprotein expression in IOVs

P-gp protein expression was determined in 200 individual samples of human placental brush border membrane. Western blot analysis of 4 selected term placentas is shown as an example in Fig. 2. The expression of P-gp ranged between 0.006 and 0.14 μg P-gp/μg total protein with a mean of 0.04 μg P-gp/μg total protein. These results demonstrate the wide range of P-gp protein expression between individual term placentas obtained from healthy pregnancies.

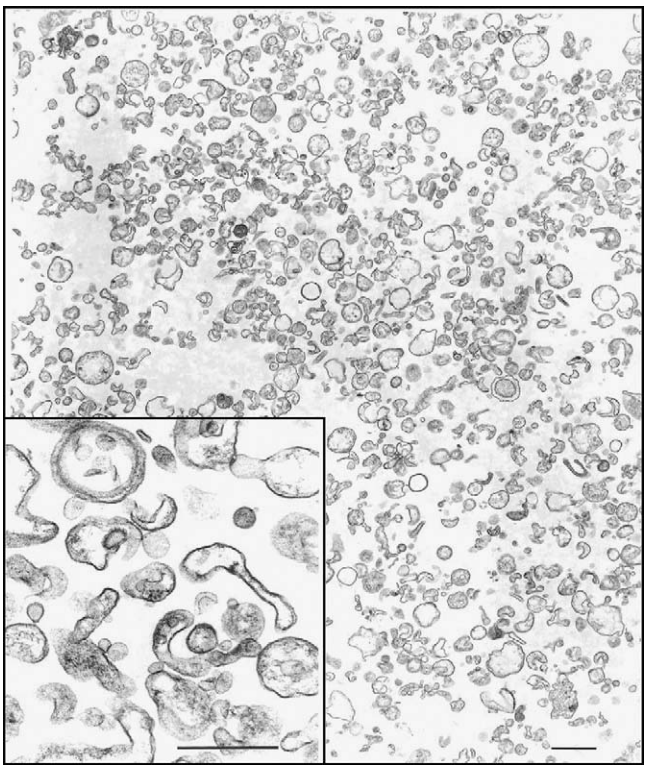

Fig. 1. Electron micrograph of brush border membrane vesicles. Vesicles were fixed in 2.5% formaldehyde and 0.03% $CaCl_2$, washed in 0.1 M cacodylate buffer and fixed in 1% OsO_4 in 0.1 M cacodylate buffer. The pellet was *en bloc* stained with 2% aqueous uranyl acetate, dehydrated in a graded series of ethanol, and embedded in Poly/Bed 812. Ultra thin sections were cut on an ultramicrotome, stained with lead citrate and examined in a Philips 201 electron microscope at 60 kV. Scale bar = 1 μm. Low magnification (62,700×, scale bar 1 μm), inset: high magnification (14,100×, scale bar 1 μm).

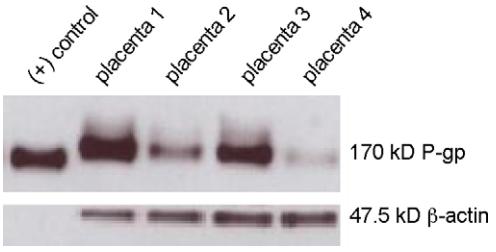

Fig. 2. P-gp protein expression in human placental brush border membranes in representative term placentas (10 μg/lane). The immunoblot was probed with the monoclonal antibody C219. Immunoreactive protein bands detected by immunoblotting were analyzed by densitometry. P-gp expression was determined as a proportion of the total amount of β-actin present per lane. Mean ± SD were calculated in 200 placentas.

3.4. P-gp-mediated transport of paclitaxel

ATP-dependent uptake of [^{3}H]-paclitaxel into IOVs was determined in 200 individual placentas. The uptake of paclitaxel ranged between 1 and 140 with a mean of 23 pmol mg protein^{-1} min^{-1}. The transport activity of P-gp in human placental IOVs was time-dependent (Fig. 3), and active transport was detected in samples within 10 s. The time course of paclitaxel uptake by IOVs indicates that it reaches equilibrium, with opposing diffusion out of the vesicles, within minutes. The ATP-dependent uptake of [^{3}H]-paclitaxel by P-gp in the IOVs exhibited classical saturation kinetics with V_{max} of 20 ± 3 pmol mg protein^{-1} min^{-1} and an apparent K_t of 66 ± 38 nM (Fig. 4A). No correlation was

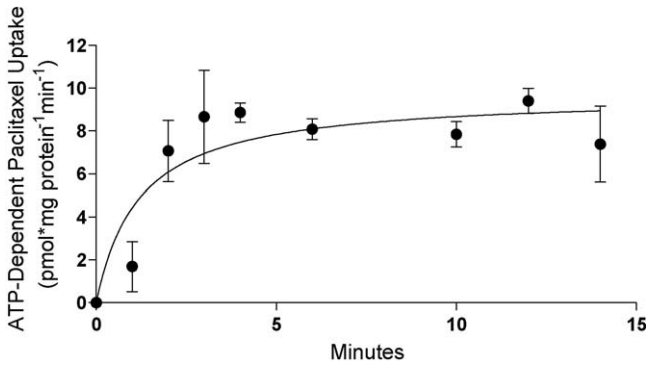

Fig. 3. Time-dependent P-gp transport activity was measured in human placental brush border membrane vesicles (n = 4, error bars represent mean ± SEM). Vesicles (0.05 mg/mL) were incubated for 1–14 min with 70 nM [^{3}H]-paclitaxel in the presence or absence of an ATP regenerating system and isolated by rapid filtration using a cell harvester. [^{3}H]-paclitaxel retention was detected using liquid scintillation counting.

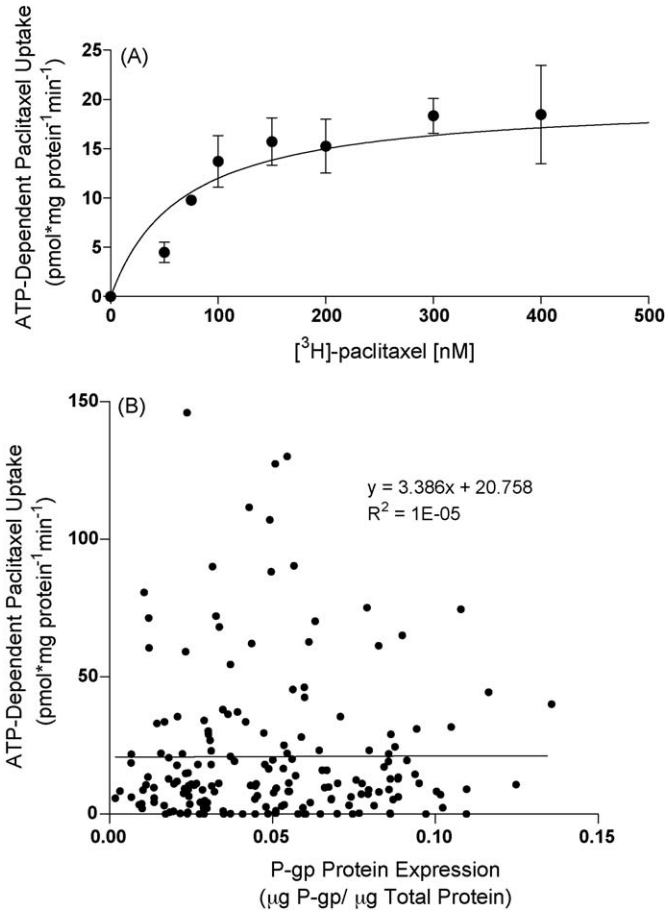

Fig. 4. (A) Saturation curve of ATP-dependent uptake of paclitaxel by placental IOVs (n = 10, error bars represent mean ± SEM). Vesicles (0.05 mg/mL) were incubated with 20–500 nM [^{3}H]-paclitaxel in the presence or absence of an ATP regenerating system and isolated by rapid filtration using a cell harvester. [^{3}H]-paclitaxel retention was detected using liquid scintillation counting. (B) Correlation between P-gp protein expression (μg P-gp/μg total protein) and ATP-dependent [^{3}H]-paclitaxel uptake in placental IOVs (n = 200). No correlation was observed between P-gp protein expression and transport activity (R^2 = 0.00001).

observed between P-gp protein expression and transport activity (R^2 = 0.00001) (Fig. 4B).

Transport of [^{3}H]-paclitaxel was inhibited by C219 (monoclonal antibody to P-gp) and by verapamil (P-gp-selective inhibitor), 85 ± 5% and 99 ± 9%, respectively, indicating paclitaxel is preferen-

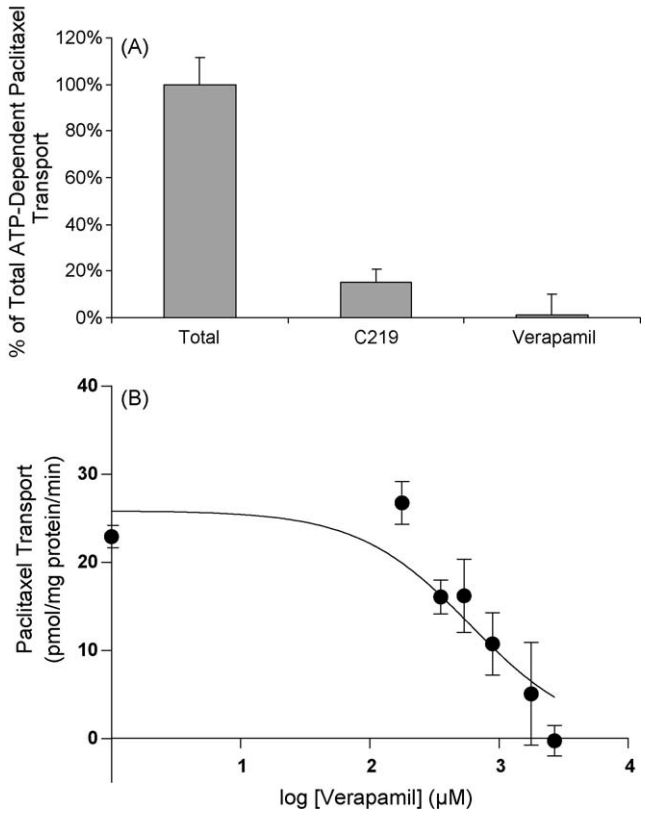

Fig. 5. Inhibition of paclitaxel transport by monoclonal antibody C219 and P-gp-selective inhibitor verapamil ($n = 4$, error bars represent mean ± SEM). (A) Vesicles (0.05 mg/mL) were incubated with C219 (0.05 mg/mL) or verapamil (2 mM). (B) Verapamil inhibited ATP-dependent transport of [^{3}H]-paclitaxel (70 nM) with a K_i of approximately 300 μM.

tially transported by P-gp (Fig. 5A). The K_i for verapamil inhibition of paciltaxel transport was approximately 300 μM (Fig. 5B).

3.5. Inhibition of paclitaxel transport by opiates

Methadone inhibited ATP-dependent transport of paclitaxel by 60% with a K_i of approximately 18 ± 3 μM (Fig. 6A). Buprenorphine inhibited ATP-dependent transport of paclitaxel by 80% with a K_i of approximately 44 ± 5 μM (Fig. 6B). Morphine inhibited ATP-dependent transport of paclitaxel by 100% with a K_i of approximately 90 ± 3 μM (Fig. 6C).

4. Discussion

The goals of this investigation were: (1) characterize the P-gp-mediated transport of its prototypic substrate, paclitaxel using preparations of placental inside-out vesicles, (2) correlate placental P-gp protein expression with its transport activity, and (3) utilize IOVs to investigate the effects of the opiates buprenorphine, methadone, and morphine on the kinetics of P-gp-mediated transport of paclitaxel.

Efflux transporters, including P-gp, have overlapping substrate specificity with members of the ABC family of transporters as well as those in other families. Accordingly, it is important to determine whether a drug is extruded by P-gp only or by more than one of the transporters localized in the apical membranes of human placenta — e.g. BCRP [18] — which has overlapping substrate specificity with P-gp [19]. Paclitaxel was transferred from the maternal to fetal circuit in the dually perfused placental lobule to a greater extent when perfused in the presence of a P-gp inhibitor, GF120918,

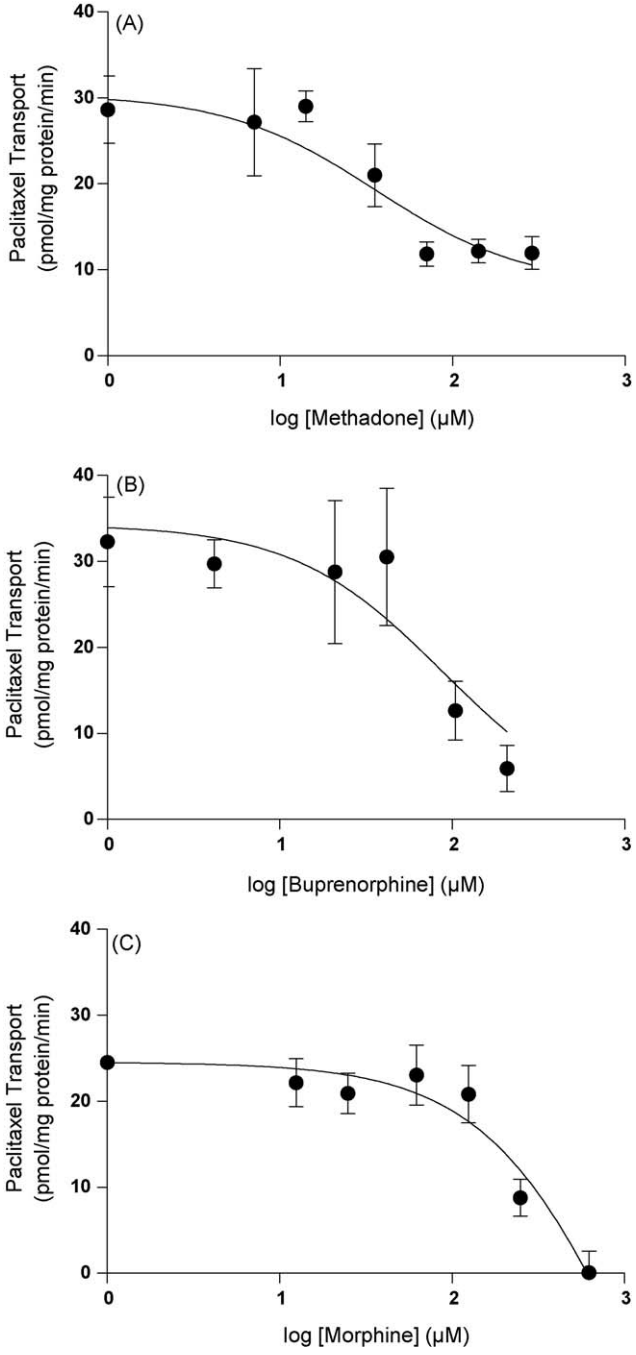

Fig. 6. Inhibition of P-gp-mediated [^{3}H]-paclitaxel transport in placental brush border membrane vesicles (0.5 mg/mL) by (A) methadone, (B) buprenorphine, and (C) morphine. (A) Methadone inhibited ATP-dependent transport of paclitaxel (70 nM) with a K_i of approximately 18 μM ($n = 8$, error bars represent mean ± SEM). (B) Buprenorphine inhibited ATP-dependent transport of paclitaxel (70 nM) with a K_i of approximately 44 μM ($n = 4$, error bars represent mean ± SEM). (C) Morphine inhibited ATP-dependent transport of paclitaxel (70 nM) with a K_i of approximately 90 μM ($n = 4$, error bars represent mean ± SEM).

providing indirect evidence that it is transported by placental P-gp [13]. It should be noted that GF120918 also inhibits the activity of BCRP; as a result verapamil was chosen as a P-gp-selective inhibitor in this investigation. Previous reports demonstrated that paclitaxel is preferentially transported by P-gp but not by BCRP or the other ABC transporters [20]. Therefore, the activity of placental P-gp and the kinetics of transport were determined using the prototypic substrate, paclitaxel.

Inside-out vesicles (IOVs) prepared from term placental brush border membranes were evaluated for their use in determining P-gp transport activity. Electron microscopy and alkaline phosphatase activity confirmed that the preparations consisted of vesicles composed of brush border membrane bilayer. Paclitaxel uptake by placental P-gp exhibited saturation kinetics and was ATP-dependent. Analysis of the data obtained revealed an apparent K_t of 66 ± 38 nM and V_{max} of 20 ± 3 pmol mg protein^{-1} min^{-1}. To the best of our knowledge, there are reports neither on the direct transport of paclitaxel by placental P-gp nor on the kinetics of its transfer. An investigation of the kinetics of paclitaxel transport by P-gp of CEM leukemia cells reported a K_t of 144 ± 56 nM in parental cells. In the same cell line, after several passages in cultures containing vinblastine (conferring multidrug resistance), the resulting cells overexpressed P-gp and revealed their transport of paclitaxel with an apparent K_t of 63 ± 46 nM [21]. Accordingly, the apparent K_t of 66 nM determined for placental IOVs is in agreement with that reported for the leukemia cell line. Therefore, the placental brush border membrane vesicle preparations characterized in this investigation provide an effective method for the identification of the drugs/medications that are substrates of P-gp as well as their interaction (inhibition) of the transport of its prototypic substrate, paclitaxel.

The second goal of this investigation was to correlate the expression of P-gp protein and its transport activity in placental inside-out vesicles. The progression of pregnancy is accompanied by changes in maternal physiology as well as developmental changes in the feto-placental unit which may include up- or downregulation of enzymes and transporters. It has been speculated that the placental expression of P-gp declines throughout gestation [22,23] (although longitudinal studies in an individual placenta are not possible), however it is unclear whether P-gp activity in fact correlates with its expression. Therefore, the protein expression of P-gp and its corresponding activity were determined in 200 individual placental preparations of IOVs. Each placenta was obtained either from term healthy pregnancies or from preterm deliveries of various etiologies. The wide range of P-gp expression between individual placentas observed in this investigation is in agreement with previous findings from our laboratory [12]. In addition to the variability in P-gp protein expression, there was no correlation between P-gp protein expression and its activity (coefficient $R^2 = 0.00001$). Similarly, a lack of correlation between P-gp expression and its activity has been reported in human lymphocytes [24]. Therefore, it can be concluded that the expression of P-gp protein in placental syncytiotrophoblast cannot be used as a predictor of its activity in the efflux of a drug/medication from the feto-placental unit to the maternal circulation.

The third goal of this report was to investigate the interaction (binding) between the two pharmacotherapeutic agents used in maintenance of the pregnant opiate addict (methadone and buprenorphine) as well as morphine and their effect on the activity of term placental P-gp in the transport of its prototypic substrate paclitaxel. Methadone, buprenorphine, and morphine decreased the transfer of paclitaxel by placental P-gp with an apparent K_i of approximately 18, 44, and 90 μM, respectively. In a previous report from our laboratory, utilizing the *ex vivo* model of dual perfusion of placental lobule (DPPL), we identified methadone, but not buprenorphine, as a substrate that is transported by P-gp.

Taken together, the data obtained in this report utilizing uptake of paclitaxel by placental IOVs indicate that morphine and methadone are P-gp substrates which compete with paclitaxel for its binding site and consequently its transport. On the other hand, buprenorphine acts as competitive inhibitor of paclitaxel binding to P-gp, although it is not transported. The apparent K_i for inhibiting paclitaxel transport determined for the three opiates used in this study were lower (higher affinity) than that determined for the known inhibitor of P-gp, verapamil (apparent K_i, 300 μM). Therefore, methadone, buprenorphine, and morphine are more potent than verapamil as inhibitors of paclitaxel transport by human placental P-gp. Interestingly, methadone caused partial (60%) inhibition of P-gp-mediated transport of paclitaxel (Fig. 6A), which could suggest the involvement of more than one binding site affecting the transport of paclitaxel and methadone. It is recognized that there are multiple drug binding sites on P-gp capable of allosteric interactions [25], and further investigation of the substrate specificity of the binding sites is needed.

P-gp transports a variety of xenobiotics, endogenous compounds, and possibly metabolic waste products from the feto-placental unit to the maternal circulation. Consequently, the extent to which a compound may interfere with P-gp (drug interactions) at the maternal–fetal interface could affect the ability of the placenta to serve as a functional barrier in limiting fetal exposure to administered medications. Drug–drug interaction represents one of the many factors which may influence biodisposition of therapeutics at the maternal–fetal interface. An earlier report of placental aromatase inhibition by methadone and buprenorphine demonstrated that the human placenta is a target for drug interactions in pregnant women under treatment with these medications [26]. However, opiate interactions with P-gp, as they relate to the development and severity of NAS, require future *in vivo* studies during pregnancy.

It is possible that genetic influences, such as variability in the MDR1 gene encoding P-gp, may contribute to the lack of correlation between protein expression and activity. It remains unknown whether MDR1 genotype plays a role in fetal exposure to opiates. Genetic variants in MDR1 have been associated with differing methadone plasma levels and maintenance dose in opiate-dependent patients [27–29], however there is no clear consensus on the role of MDR1 genotype in the transport of methadone or interaction with other opioids.

In summary, our data indicate that a method has been validated for determining the activity of placental brush border membranes efflux transporter P-gp using paclitaxel as a prototypic substrate. This method allowed the identification of methadone, buprenorphine, and morphine as inhibitors of paclitaxel transport by human placental P-gp, as compared to that of the classic P-gp inhibitor verapamil. This method is a useful tool in identifying the medications that are substrates of P-gp and consequently the extent of their transfer to the fetal circulation during pregnancy.

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