

Interaction of drugs of abuse and maintenance treatments with human P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2)

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Abstract

Drug interaction with P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) may influence its tissue disposition including blood–brain barrier transport and result in potent drug–drug interactions. The limited data obtained using *in-vitro* models indicate that methadone, buprenorphine, and cannabinoids may interact with human P-gp; but almost nothing is known about drugs of abuse and BCRP. We used *in vitro* P-gp and BCRP inhibition flow cytometric assays with hMDR1- and hBCRP-transfected HEK293 cells to test 14 compounds or metabolites frequently involved in addiction, including buprenorphine, norbuprenorphine, methadone, ibogaine, cocaine, cocaethylene, amphetamine, *N*-methyl-3,4-methylenedioxyamphetamine, 3,4-methylenedioxyamphetamine, nicotine, ketamine, Δ^9 -tetrahydrocannabinol (THC), naloxone, and morphine. Drugs that *in vitro* inhibited P-gp or BCRP were tested in hMDR1- and hBCRP-MDCKII bidirectional transport studies. Human P-gp was significantly inhibited in a concentration-dependent manner by norbuprenorphine > buprenorphine > methadone > ibogaine and THC. Similarly, BCRP was inhibited by buprenorphine > norbuprenorphine > ibogaine and THC. None of the other tested compounds inhibited either transporter, even at high concentration (100 μ M). Norbuprenorphine (transport efflux ratio \sim 11) and methadone (transport efflux ratio \sim 1.9) transport was P-gp-mediated; however, with no significant stereo-selectivity regarding methadone enantiomers. BCRP did not transport any of the tested compounds. However, the clinical significance of the interaction of norbuprenorphine with P-gp remains to be evaluated.

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Introduction

The responses of individuals to drugs are influenced by a combination of pharmacokinetic and pharmacodynamic factors. The great variations in clinical responses to both abuse drugs and maintenance treatments from one individual to another may result in toxicity. Clinical reports clearly described wide dose–response ranges (Kadiev *et al.* 2008), as illustrated by the highly variable methadone (Met) doses

required to accurately maintain heroin addicts (Levrant *et al.* 2008). Highly polymorphic liver and intestine drug-metabolizing enzymes like cytochrome P450 (CYP) are generally believed to contribute to this variability in humans, also resulting in potent drug–drug interactions with other xenobiotics (Maurer *et al.* 2006). However, pharmacodynamic factors like the induction of tolerance to opioid effects also contribute greatly to drug variability.

Studies on drug transport involving ATP-binding cassette (ABC) transporters including P-glycoprotein (ABCB1, MDR1, P-gp) and breast cancer resistance protein (ABCG2, BCRP, MXR) are becoming more numerous. P-gp and BCRP are the major efflux transporters at the blood–brain barrier (BBB) (Schermann, 2005). We recently showed that P-gp and BCRP are the

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main ABC transporters at the human BBB (Dauchy et al. 2008). There are also more publications on the ABC-mediated transport of some drugs of abuse *in vitro* and in animal models. Met has been shown to inhibit P-gp and be transported by it *in vitro* (Crettol et al. 2007; Störmer et al. 2001) and *in vivo* in rodents (Dagenais et al. 2004; Wang et al. 2004). Suzuki et al. (2007) described the P-gp-mediated transport of buprenorphine (Bup) at the mouse BBB but this was not demonstrated for human placental lobules (Nekhayeva et al. 2006) or caco-2 cells (Hassan et al. 2009). The transport of morphine by human P-gp *in vitro* remains controversial (Feng et al. 2008; Schinkel et al. 1995; Wandel et al. 2002) while naloxone (Nal) was not shown to be a P-gp substrate (Mahar Doan et al. 2002). However, the basolateral-to-apical polarized transport of cocaine across the endothelial cells of bovine brain microvessels suggests that cocaine is transported by bovine P-gp (Raje et al. 2003). In contrast, no evidence has been found for the transport of D-amphetamine, N-methyl-3,4-methylenedioxyamphetamine (MDMA), or 3,4-methylenedioxyamphetamine (MDA) by P-gp *in vitro* (Bertelsen et al. 2006; Zhu et al. 2008), and nicotine does not seem to be a substrate of human P-gp (Wang et al. 2005).

Our analysis of the published literature shows that a wide range of very different experimental models have been used, which limits any extrapolation to the clinical setting. Several models are not specific to P-gp. Neither can data be compared directly or with reference to known P-gp substrates or inhibitors. Differences between species are rarely considered, so that a human-specific model is needed (Takeuchi et al. 2006). Finally, data on the interactions of drugs of abuse with human BCRP are lacking, except for the major cannabinoids from marijuana (Bonhomme-Faivre et al. 2008; Holland et al. 2006, 2007; Zhu et al. 2006a).

The impact of P-gp on the distribution of morphine (Kharasch et al. 2003) and Met was investigated in humans. The inhibition of P-gp enhanced the intestinal absorption of morphine and Met but had no significant pharmacodynamic effect at the BBB (Kharasch et al. 2004). P-gp gene polymorphism has recently been proposed to contribute in part to the variations in Met dose requirement in humans, suggesting that P-gp-mediated Met transport may influence its final activity (Levrán et al. 2008). Moreover, drug interaction with transporters is a major concern as addicts may use all kinds of medications including antiretroviral, antipsychotic, antidepressant, and anxiolytic drugs that may be P-gp or BCRP substrates, thus resulting in deleterious drug–drug interactions (Antoniu & Tseng, 2002; Pal & Mitra, 2006).

In the present study we used a reproducible, validated, and highly specific *in-vitro* model to determine whether the common drugs of abuse and maintenance treatments are transported by, or inhibit, human P-gp and BCRP, leading to drug–drug interactions. We first used flow cytometric inhibition assays with hMDR1 and hBCRP-transfected HEK293 cells. The transport of the drugs that were found to interact significantly with transporters was then studied using bidirectional transport studies with hMDR1- and hBCRP-MDCKII cells.

Method

Chemicals

Bup hydrochloride and norbuprenorphine (norBup) were generously provided by Schering-Plough SA (USA). Cocaine hydrochloride, cocaethylene, MDMA, MDA, Δ^9 -tetrahydrocannabinol (THC), nicotine, ketamine hydrochloride, ibogaine hydrochloride (Ibo) and Nal hydrochloride dehydrate, calcein-AM, calcein, rhodamine-123 (Rh-123), Lucifer Yellow (LY) and mitoxantrone (MXR) were from Sigma-Aldrich (France). D-amphetamine sulfate was a gift from Marie-Claude Menet (Paris Descartes University). Morphine hydrochloride and racemic Met [(R,S)-Met] were from Francopia (France). PSC833 (valspodar) was a gift from Novartis (Switzerland). Fumitremorgine C (FTC) and pheophorbide-a (Ph-a) were kindly provided by Dr Robert W. Robey (National Institutes of Health, USA). [O-methyl- 3 H]buprenorphine (3 H]Bup; 80–85 Ci/mmol) was from American Radiolabelled Chemicals (USA) and 3 H]prazosin (86.8 Ci/mmol) was from PerkinElmer Sciences (Belgium).

Cell culture

HEK293-hMDR1, HEK293-hBCRP (482R) and HEK293-pcDNA (control cells) were provided by Dr Robert W. Robey. They were maintained in Eagle's Minimum Essential Medium (ATCC, USA) with 2 mg/ml G418 (geneticin) from Invitrogen (France) to enforce the selection of stable transfectants. MDCKII-hMDR1, MDCKII-hBCRP and MDCKII-parental cells were generously provided by Dr Alfred Schinkel (National Cancer Institute, The Netherlands) and were grown in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen). The media contained 10% heat-inactivated fetal bovine serum, glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). Cells were maintained in humidified 95% air/5% CO₂ at 37 °C and passaged twice a week.

Whole cell lysates and Western blotting

Western blotting experiments were conducted as previously described (Dauchy *et al.* 2008). Briefly, MDCKII and HEK293 cultures (90% confluent) in 75-ml culture flasks were lysed with buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate, 0.5% Triton X-100 and Complete™ protease inhibitor complex, Roche Diagnostics, France]. Protein samples were separated on 8% (P-gp) or 10% (BCRP) SDS-PAGE gels and electrotransferred to nitrocellulose membranes (Hybond, Amersham, France). The membranes were immunoblotted with anti-P-gp C219 (diluted 1:200), or anti-BCRP BXP-21 antibodies (1:200) (Abcam, UK) for 2 h at room temperature, then incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:10000) (Amersham, UK). The membranes were then exposed to the Amersham ECL system. Signals were revealed with the Bio-Rad ChemiDoc® XRS imaging device (Bio-Rad, USA).

Flow cytometric efflux assays

P-gp inhibition

This assay was performed essentially as described by Robey *et al.* (2001). Briefly, log phase HEK293-transfected cells were harvested and suspended in incubation buffer (phenol red-free DMEM) supplemented with 10% FBS (10⁶ cells/vial; 1 ml final volume). Specific inhibitors or test compounds were added during the accumulation phase. Vials were vortexed and the appropriate fluorescent probe added to the cell suspension. The vials were kept for 30 min at 37 °C for the cells to take up the fluorescent probes. They were then washed (5 ml ice-cold PBS), centrifuged, and suspended in 1 ml incubation buffer containing inhibitors or test compounds at the same concentration as in the accumulation phase. All vials were then incubated at 37 °C for 1 h to allow the efflux of fluorescent dye by transporters. Finally, cells were washed, suspended in 1-ml ice-cold PBS and kept on ice until used for flow cytometry. The P-gp inhibition assay with HEK293-hMDR1 cells required 1 μM calcein-AM or 1.5 μM Rh-123 as probe. No efflux phase was required for the calcein-AM assay, as this probe is rapidly hydrolysed by intracellular esterases to fluorescent calcein, which is not transported by P-gp. The positive control of P-gp inhibition was 5 μM PSC833.

BCRP inhibition

BCRP inhibition was assayed using HEK293-hBCRP cells as described above, using 10 μM MXR or 1 μM

Ph-a. The specific BCRP inhibitor was 10 μM FTC. Rh-123 transport by BCRP-overexpressing cells was also investigated to ensure that the HEK-hBCRP cells produced wild-type BCRP (482R) and not a BCRP mutant (482T or 482G) that transports Rh-123 (Robey *et al.* 2003). Ph-a is also specifically transported by the 482R wild-type (Robey *et al.* 2004).

Intracellular fluorescence was measured using a Becton-Dickinson (USA) FACSCAN flow cytometer equipped with a 488-nm argon laser: 650-nm band-pass filter for MXR and Ph-a; and a 530-nm band-pass filter for calcein and Rh-123. Fifteen thousand events were collected for each sample. Cell debris was eliminated by gating on forward *vs.* side scatter. The maximal DMSO and ethanol concentrations were 1 and 0.5% (v/v); they did not affect BCRP activity. Vehicle concentrations in control and tested vials were always identical. Blank histograms for determining cell autofluorescence were prepared with cells in PBS and vehicle alone. The geometric mean (arbitrary units) of flow cytometry histograms was obtained with CellQuest® software (USA). The accumulation index reflected the inhibition potential of the tested drugs:

$$\text{Accumulation index} = \frac{\text{geo mean}_{\text{molecule x}}}{\text{geo mean}_{\text{without inhibitor}}}$$

There were 100 μM of drug used for the initial inhibition tests. Lower concentrations were tested when flow cytometry indicated cell toxicity as for THC. Only drugs which inhibited the carrier-mediated transport of the fluorescent substrate at 100 μM were considered to be inhibitors and were further investigated. Drugs that were potent P-gp or BCRP inhibitors were also tested in HEK293-pcDNA cells to ensure that they did not induce transporter-independent intracellular accumulation of fluorophore. None of the drug (100 μM) caused auto-fluorescence in the absence of fluorescent dye at the instrument settings used.

Bidirectional transport

No transport studies were done with HEK293 cells because they adhered poorly in the two-compartment system. Molecules that inhibited transporters in the flow activated cell sorting (FACS) study were used when possible for bidirectional transport studies across monolayers of transfected MDCKII cells to test their transport by P-gp or BCRP. Cells were seeded at 3 × 10⁵ cells/cm² onto 12-well Corning Costar Transwell® polycarbonate membranes (ATGC, France) and formed monolayers within 3 d. The culture medium was changed once, the day before experiment. Transport was measured in HBSS containing CaCl₂ and MgCl₂, 1 mM pyruvate and 10 mM

Hepes (Invitrogen) in both compartments: apical-to-basolateral ($A > B$) and basolateral-to-apical ($B > A$). The culture medium was replaced with transport buffer with or without transport inhibitor in the 1 h pre-incubation. P-gp was inhibited with $5 \mu\text{M}$ PSC833 and BCRP with $10 \mu\text{M}$ FTC. MDCKII-hBCRP cells were systematically treated throughout with $2 \mu\text{M}$ PSC833 to block canine P-gp activity and enhance BCRP transport specificity. After pre-incubation, the donor compartment was filled with transport medium containing the test compound with/without inhibitors, plus $50 \mu\text{M}$ LY to check monolayer integrity. (R,S)-Met was tested at $5 \mu\text{M}$ and norBup at $10 \mu\text{M}$ to ensure detection in the opposite compartment: the limit of quantification (LOQ) of the analytical procedure. Bovine serum albumin (BSA, 1%) was added to [^3H]Bup ($0.2 \mu\text{Ci/ml}$), or up to $5 \mu\text{M}$ unlabelled Bup to avoid unspecific binding of Bup to the polystyrene plate. Monolayer efflux was stopped after 90 min for P-gp cells and 3 h for BCRP cells. The concentrations of the tested compounds and LY in the receiver compartment were determined. The transepithelial electrical resistance (TEER) was measured before and after the experiment with a Millicell[®] ERS ohm-meter (Millipore, France). The apparent permeability coefficient P_{app} was calculated from:

$$P_{\text{app}}(\text{nm/s}) = (dQ_r/dt)/(A \times C_0) \quad (1 \text{ nm/s} = 1 \times 10^{-7} \text{ cm/s}),$$

where dQ_r/dt is the amount accumulated in the receiver compartment over time; A is the area of the cell monolayer; and C_0 is the initial concentration ($t=0$) of the test solution. Data are given as the average P_{app} ($\text{nm/s} \pm \text{s.d.}$) from 6–9 monolayers. Monolayer integrity was considered as acceptable when LY P_{app} was $\leq 20 \text{ nm/s}$ and $\text{TEER} > 200 \Omega \text{ cm}^2$. The transport ratio was determined by dividing the mean $P_{\text{app}(B > A)}$ by $P_{\text{app}(A > B)}$. Transport-mediated efflux was suggested when the transport ratio was > 1.5 (Polli et al. 2001). P_{app} in the same direction in the presence and absence of inhibitor had also to be significantly different. Experimental conditions were validated using the P-gp substrate Rh-123 with MDCKII-hMDR1 cells, and the BCRP substrate [^3H]prazosin with MDCKII-BCRP cells. Bidirectional studies were also performed on MDCKII-parental clones to estimate the involvement of basal canine transporters.

Analytical procedure

Rh-123 and LY were quantified using a Cytofluor series 4000 fluorescence plate reader (Applied Biosystem, France). [^3H]prazosin and [^3H]Bup were counted in a Tri-Carb model 1900 TR (Packard, France). Bup,

norBUP and (R/S)-Met samples were frozen at -20°C , until quantified by LC-MS/MS. Bup and norBup were analysed as previously described (Pirnay et al. 2006). Separation of Met enantiomers was achieved using a Cyclobond I-2000 HP-RSP chiral column ($250 \text{ mm} \times 4.6 \text{ mm i.d.}$, $5 \mu\text{M}$, Thermo Electron Corporation, USA) and single ion storage detection. LOQ was defined as 1 ng/ml for both Met enantiomers.

Statistical analysis

Unpaired two-tailed Student's t tests were used to determine statistical significance between groups. The mean accumulation index had to be significantly > 1 ($p < 0.05$) to assess transporter inhibition potency by flow cytometry ($n=3$ minimum). Bidirectional transport data are presented as means \pm s.d. for 6–9 independent values.

Results

P-gp and BCRP in HEK293 and MDCK cells

Western blots of the whole HEK293-MDR1 and MDCKII-MDR1 cell lysates showed a major band at $\sim 170 \text{ kDa}$, indicating high P-gp concentrations (Fig. 1a). Homogenates of MDCKII-MDR1 and parental MDCKII cells also gave a second more weakly stained band at $\sim 140 \text{ kDa}$, which could be canine P-gp, as previously reported (Taub et al. 2005). Western blotting of HEK293-BCRP and MDCKII-BCRP cell homogenates using anti-BXP-21 anti-BCRP antibodies revealed an intense band at $\sim 70 \text{ kDa}$, corresponding to a high BCRP concentration. A second less intense band at a higher molecular weight was assessed. The MDCKII and HEK293 parental cells showed no band at $\sim 70 \text{ kDa}$ (Fig. 1b).

Flow cytometric efflux assay

The inhibition potency of each tested compound towards human P-gp and BCRP was evaluated using specific fluorescent dyes that were transported by either P-gp or BCRP. HEK293-pcDNA cells accumulated much more fluorescent dye than did transporter-transfected cells, comparable to histograms obtained in the presence of inhibitors.

P-gp inhibition study

The capacity of a compound to inhibit P-gp was evaluated by measuring either the uptake of calcein-AM or the efflux of Rh-123 by HEK293-MDR1 cells in the presence or absence of the compound at $100 \mu\text{M}$. P-gp-mediated calcein-AM transport was significantly inhibited as follows, starting with the most potent

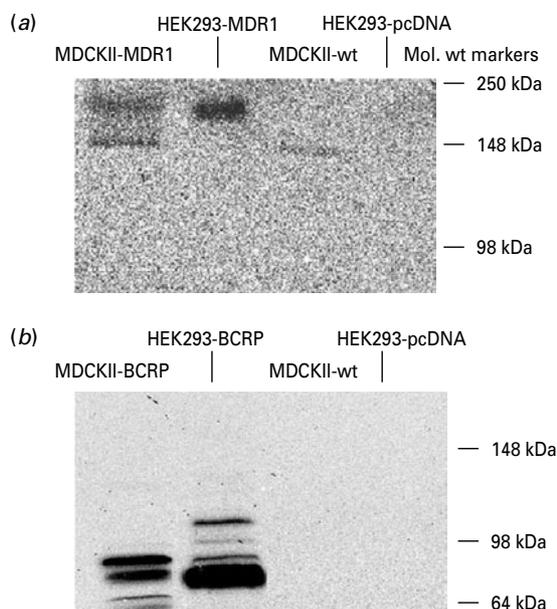


Fig. 1. (a) P-gp and (b) BCRP expression in several MDCK and HEK293 cell lines. Whole-cell lysate (10 μ g protein) samples were size fractionated in (a) 8% SDS-PAGE or (b) 10% SDS-PAGE gels and electrotransferred to nitrocellulose membranes. The membranes were then immunoblotted with (a) C-219 anti-P-gp or (b) the BXP-21 anti-BCRP antibodies, and protein-antibody interactions revealed by chemiluminescence.

compound: norBup > Bup > Met > Ibo. Their effect at 100 μ M was comparable to that of the powerful P-gp inhibitor, 5 μ M PSC833. THC also inhibited P-gp-mediated transport, but to a lesser extent. It was about 10-fold less potent than 5 μ M PSC833.

Then, we measured the inhibitory potency of norBup, Bup, Met and Ibo at lower concentrations and found a concentration-effect relationship for all of them (Fig. 2). None of the other tested drugs (at 100 μ M) had any significant effect on P-gp activity. Rh-123 was also used as a fluorescent P-gp substrate. Although PSC833 decreased the efflux of Rh-123 by P-gp ~150-fold, Met was the only compound that slightly inhibited P-gp-mediated transport of Rh-123 in a concentration-dependent manner. None of the other compounds had any significant influence on P-gp-mediated Rh-123 transport.

BCRP inhibition study

The capacity of a drug to inhibit BCRP was also evaluated by measuring the efflux of BCRP substrates, MXR and Ph-a, in BCRP-HEK293 cells, with or without 100 μ M concentrations of drugs of abuse and maintenance treatments. Bup was the most potent inhibitor of

BCRP-mediated MXR efflux, while 50 μ M concentrations of norBup > Ibo and THC were much less potent. Bup, norBup and Ibo actions were concentration-dependent. Bup was the most potent BCRP inhibitor when tested with MXR (Fig. 3); however, it did not inhibit BCRP-mediated transport of Met or several other compounds. These results were confirmed using Ph-a as a probe of BCRP activity. The inhibition profiles were quite similar to those obtained using MXR (Fig. 3).

Bidirectional transport study

The actions of compounds that significantly inhibited P-gp and/or BCRP by flow cytometry were studied on monolayers of MDR1- and BCRP-MDCKII-transfected cells. The transport ratios of norBup, S-Met and R-Met in untransfected MDCKII-parental cells were: 1.7 (norBup), 1.2 (S-Met) and 1.1 (R-Met). P-gp- and BCRP-mediated transport in MDCK-transfected cell lines was validated using Rh-123 as substrate for P-gp and [³H]prazosin for BCRP (Table 1). The specificity of transport was assessed using 5 μ M PSC833 to inhibit P-gp and 10 μ M FTC to inhibit BCRP.

P-gp transport study

P-gp-mediated transport of Bup, norBup and Met was tested in MDCK-MDR1 cells. Both R-Met and S-Met were poorly transported by P-gp, with no significant enantio-selectivity. Met polarized transport was completely blocked by PSC833.

Bup bidirectional transport was difficult to assess because of its strong unspecific binding to the polystyrene plate or to the cell monolayer. The mass balance at the end of incubation revealed that ~30% of Bup was not recovered. Using high concentrations of unlabelled Bup with radiolabelled Bup or BSA did not significantly improve this mass balance. Thus, P_{app} values regarding Bup should be carefully interpreted. We were never able to show any polarized Bup transport or any influence of specific inhibitors on Bup P_{app} in similar conditions when testing the same A > B or B > A directions.

In contrast, norBup had a surprisingly high relative P_{app} in the A > B direction, close to that of CNS compounds like Met. norBup transport through the MDR1-MDCK monolayers was strongly influenced by P-gp, with a transport ratio of ~11. Hence, norBup is a good substrate for P-gp in this model.

BCRP transport study

BCRP-mediated transport of the potent BCRP inhibitors Bup and norBup was investigated. No significant

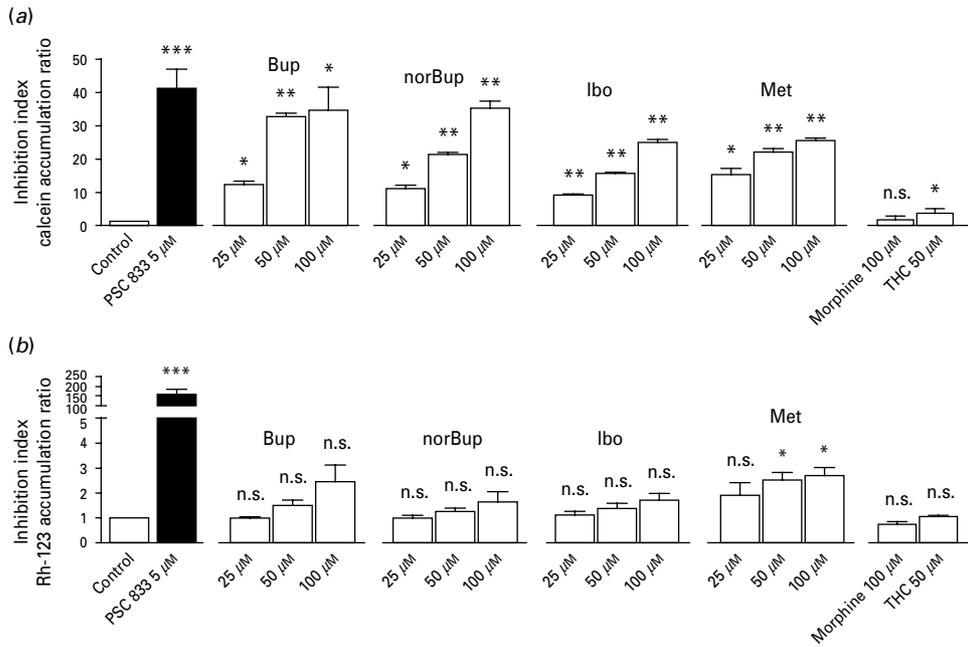


Fig. 2. Inhibition of P-gp by norBup, Bup, lbo, Met, morphine and THC. Data were obtained by flow cytometric assay using (a) calcein-AM or (b) Rh-123 as probes for P-gp-mediated efflux from HEK293-MDR1 cells. The accumulation index was calculated by dividing the intracellular accumulation of fluorescent probe in the presence of test compound by the accumulation in the absence of inhibitor. PSC833 was used as a positive control for P-gp inhibition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s., no significant difference.

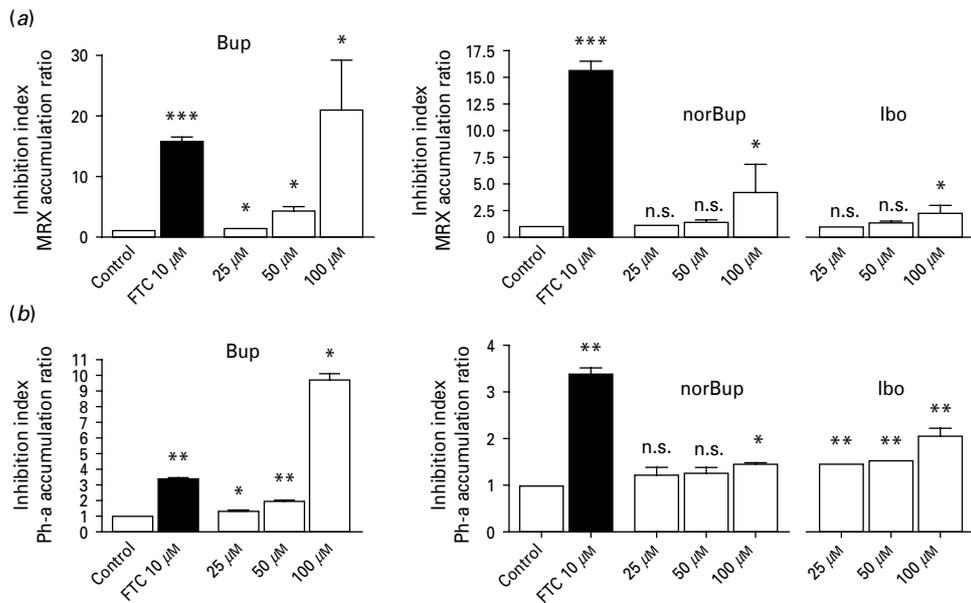


Fig. 3. Inhibition of BCRP by Bup, norBup and lbo. Data were obtained by flow cytometric assay using (a) MXR or (b) Ph-a as probes for BCRP-mediated efflux from HEK293-BCRP cells. The accumulation index was calculated by dividing the intracellular accumulation of fluorescent probe in the presence of the test compound by the accumulation in the absence of the inhibitor. FTC was used as a positive control for BCRP inhibition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s., no significant difference.

Table 1. Bidirectional transport across MDR1- and BCRP-MDCKII transfected cells

Compound	Treatment	MDCK-MDR1			MDCK-BCRP		
		$P_{app(A>B)}$ (nm/s)	$P_{app(B>A)}$ (nm/s)	Transport ratio	$P_{app(A>B)}$ (nm/s)	$P_{app(B>A)}$ (nm/s)	Transport ratio
Rh-123 10 μ M	Control	8.3 \pm 0.4	196.3 \pm 22.1***	3.9	23.5 \pm 0.6	25.5 \pm 2.3	1.1
	Inhibition	9.0 \pm 0.8	35.8 \pm 7.5		25.8 \pm 2.4	21.3 \pm 1.6	0.8
[³ H]Prazosin 5 μ M	Control				131.6 \pm 5.8	376.3 \pm 6.6***	2.9
	Inhibition				234.1 \pm 13.9	218.7 \pm 22.8	0.9
Bup 2 nM + 1 % BSA	Control	148.7 \pm 6.8	166.0 \pm 3.2	1.1	134.2 \pm 19.2	134.3 \pm 4.9	1.0
Bup 5 μ M	Inhibition	155.3 \pm 15.2	159.7 \pm 8.4	1.0	144.4 \pm 4.6	131.5 \pm 9.6	0.9
	Control	105.3 \pm 19.3	107.6 \pm 32.0	1.0	153.4 \pm 28.1	150.3 \pm 23.7	1.0
NorBup 10 μ M	Inhibition	103.5 \pm 23.3	113.9 \pm 45.5	1.1	94.8 \pm 15.0	120.9 \pm 5.2	1.3
	Control	52.3 \pm 2.0	594.2 \pm 55.5***	11.3	149.0 \pm 7.6	153.6 \pm 7.2	1.0
(R)-Met 5 μ M	Inhibition	219.5 \pm 53.5	208.7 \pm 16.8	1.0	127.0 \pm 3.7	144.9 \pm 9.8	1.1
	Control	62.5 \pm 7.2	117.8 \pm 19.7**	1.9			
(S)-Met 5 μ M	Inhibition	96.8 \pm 5.0	104.9 \pm 19.9	1.1			
	Control	62.2 \pm 1.2	118.7 \pm 10.8*	1.9			
	Inhibition	114.4 \pm 31.9	102.0 \pm 25.4	0.9			

Data are presented as means P_{app} (nm/s) \pm s.d. $n = 6-9$. Transport ratio was obtained from $P_{app(B>A)}/P_{app(A>B)}$.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

polarized transport through the cell monolayer was measured.

Discussion

The active transport of drugs across physiological barriers like the gut and the BBB is now recognized as a source of variability in the pharmacological responses of individuals to these drugs. Even if drugs of abuse are psycho-active compounds that readily cross biological membranes, they may also be actively transported, or may even inhibit transporter proteins. P-gp and BCRP are now considered to be involved in the active efflux system at the BBB that limits the entry of drugs into the brain. However, only few data have been published on P-gp-mediated or BCRP-mediated transport of drugs of abuse and maintenance treatments, only focusing on opiates, cannabinoids, and Met. Moreover, heterogeneous experimental models and species were used, making it difficult to reach any definitive conclusion regarding their actions in humans.

We first tested drugs in parallel conditions to determine whether they are inhibitors of human P-gp and BCRP. The capacity of drugs to inhibit P-gp was assessed by flow cytometry using two fluorescent P-gp substrates, calcein-AM and Rh-123. Previous studies on MDR1-MDCKII cells demonstrated that highly permeable compounds inhibit transporters, but

provided no evidence of transport across cell monolayers. They also established that the calcein-AM assay is much more likely to detect interactions with P-gp than are bidirectional studies (Polli *et al.* 2001). This inhibition screening is usually performed at high concentrations (100 μ M) that are in no way related to what might happen with the clinically relevant concentrations that could occur in humans. Only compounds that significantly inhibited P-gp and BCRP-mediated transport as measured by flow cytometry were investigated in bidirectional studies on MDCKII cells.

Studies with calcein-AM showed that P-gp was only inhibited by Ibo, and to a lesser extent by THC. None of the other compounds tested at 100 μ M (D-amphetamine, MDMA, MDA, cocaine, cocaethylene, nicotine, ketamine, Nal, and morphine) had any significant effect. The inhibition of P-gp by cannabinoids from marijuana was investigated by Holland *et al.* (2006) using Rh-123 to probe P-gp function. They found that THC did not inhibit P-gp, in agreement with our findings with Rh-123 as P-gp probe. But we did find that 50 μ M THC slightly inhibited P-gp-mediated calcein-AM transport, as reported by Zhu *et al.* (2006a) using another cell model. Thus, our results showed that Rh-123 is a good P-gp substrate, although not suitable for use alone to screen for P-gp inhibition as false-negative results (as for THC) are possible. By contrast, several P-gp substrates should be used before coming to any conclusion on the inability of a given

drug to inhibit P-gp. The conflicting results obtained with the two fluorescent probes may be due to different P-gp substrate-binding sites (Martin *et al.* 2000). P-gp inhibitors preferentially interact with the calcein-AM binding site of P-gp. However, the clinical relevance of P-gp inhibition by THC is difficult to assess since concentrations that inhibit P-gp *in vitro* are far higher than plasma THC concentrations usually observed in patients (peak concentrations of about 77 ng/ml–250 nm) (Ohlsson *et al.* 1980). Hence, THC is unlikely to inhibit P-gp at the human BBB. It was recently shown that THC bioavailability is greater in P-gp-deficient than in wild-type mice (Bonhomme-Faivre *et al.* 2008), suggesting that THC is efficiently transported by murine intestinal or biliary P-gp. We also find that THC slightly inhibited BCRP, in agreement with Holland *et al.* (2007). Thus the impact of the interaction of cannabinoids with ABC transporters remains important.

We assessed the capacity of maintenance therapies (Met and Bup) and norBup, the main active Bup metabolite, to inhibit P-gp. Met was found to be a powerful inhibitor of P-gp-mediated calcein-AM transport, as previously reported for Rh-123 in caco-2 cells (Störmer *et al.* 2001). Met is also a good substrate of murine P-gp, its concentration in the brains of P-gp-deficient *Mdr1a*^{-/-} mice is 15- to 23-fold higher than in the brains of *Mdr1a*^{+/+} mice (Dagenais *et al.* 2004; Wang *et al.* 2004) for (R)-Met and (S)-Met, respectively. Met pharmacokinetics in rats and humans have been extensively described as altered by P-gp during its absorption and distribution phases (Bouer *et al.* 1999; Crettol *et al.* 2008; Kharasch *et al.* 2004; Levran *et al.* 2008; Ortega *et al.* 2007; Rodriguez *et al.* 2004). P-gp-mediated transport significantly alters the pharmacological response to Met, and genetic variants influence the required Met dose to prevent withdrawal symptoms and relapse in maintained patients (Levran *et al.* 2008). Transplacental Met transfer through human lobules is also increased by the P-gp inhibitor GF120918 (Nanovskaya *et al.* 2005). Studies with our *in-vitro* models clearly showed Met transport by human P-gp, while Met's ability to inhibit P-gp was confirmed using both fluorescent P-gp substrates. However, Met efflux transport ratio was only ~2, far below Met brain concentration ratio in P-gp-deficient mice (Dagenais *et al.* 2004; Wang *et al.* 2004), but close to that observed with another human hMDR1-LLCPK cell line over-expressing P-gp (Crettol *et al.* 2007). Murine P-gp may therefore be a better transporter of Met than human P-gp. Moreover, we detected no enantio-selective P-gp-mediated transport of Met in MDCK-MDR1 cells, in contrast to previous results showing a slightly better

transport of S-Met by human P-gp (Crettol *et al.* 2007). Quinidine (600 mg p.o.) increases the CNS effects of loperamide in humans, independently from changes in its plasma concentrations (Sadeque *et al.* 2000). This indicates that quinidine efficiently inhibits P-gp at the BBB. Kharasch *et al.* (2004) used a similar clinical design (infusion of 600 mg quinidine for 5 min) to demonstrate that P-gp does not change the pharmacodynamic effects of intravenous Met. This agrees with our data, since Met was shown to be a poorer substrate of human P-gp than loperamide (high efflux ratio ~10 in transport studies using MDCK-MDR1 cells in the same experimental conditions as ours) (Mahar Doan *et al.* 2002). We have also shown that Met does not interact with human BCRP.

We studied the interactions of Bup and norBup with ABC transporters, since P-gp and/or BCRP may modulate their pharmacological activity or toxicity. There are very few published data on the interaction of Bup with ABC transporters. Bup is not transported by P-gp on human placental lobules (Nekhayeva *et al.* 2006), or by the P-gp on caco-2 cells (Hassan *et al.* 2009), although some author have suggested that it is transported by P-gp at the mouse BBB (Suzuki *et al.* 2007). We found no transport of Bup by P-gp or BCRP, although Bup strongly inhibited P-gp at high extracellular concentrations. The plasma concentrations of Bup during maintenance treatment are 1–5 ng/ml (2–11 nM), far below the concentration that inhibits P-gp *in vitro*. Bup may thus not alter the concentration or effect of any co-administered drug that is a P-gp substrate.

We showed that norBup, the main active *N*-dealkylated Bup metabolite, is a very good P-gp substrate, with a transport efflux ratio of ~11. This value is close to the transport efflux ratio of loperamide. Inhibition of P-gp leads to a huge increase in the CNS effects of this drug in humans (Sadeque *et al.* 2000). Similarly, Yassen *et al.* (2007) have suggested that norBUP is actively transported by P-gp because of its particular pharmacokinetic–pharmacodynamic profile. Thus, given its substantial P-gp-mediated transport, we may question the exact role of norBup *in vivo* in comparison to Bup in both therapeutic and toxic conditions. norBup has always been believed to make only a minor contribution to clinical effects, as it has a lower intrinsic activity than Bup (Huang *et al.* 2001; Ohtani *et al.* 1995), given that the plasma concentrations of the two molecules are similar in humans treated with Bup. However, we believe that this should be re-examined in the light of the finding that P-gp-mediated transport of norBup is greater than that of Bup. Similarly, as Bup is a partial opioid agonist with 'ceiling respiratory

effects' (Walsh *et al.* 1994), norBup contribution to Bup-attributed respiratory depression *in vivo* has been questioned (Hreiche *et al.* 2006; Mégarbane *et al.* 2006a). However, CYP3A induction, which enhances norBup concentrations in plasma, did not increase BUP-related respiratory effects in rats (Hreiche *et al.* 2006). Thus, BBB permeability to norBup remains controversial; however, our results may suggest that norBup passage through the BBB could be decreased by P-gp. Accordingly, once P-gp is inhibited, norBup concentrations in the brain could be dramatically increased, contributing to Bup toxicity. Combination of P-gp inhibitors with Bup may thus represent a real concern, leading to clinically relevant drug–drug interactions. Nevertheless, as benzodiazepines are not considered to be significant P-gp inhibitors, it is unlikely that a P-gp-related mechanism can be proposed to explain Bup-related respiratory toxicity commonly observed in drug addicts due to a combination of Bup and benzodiazepine (Mégarbane *et al.* 2006b). Moreover, the plasma concentrations usually observed in maintained patients (about 1.10 ng/ml–2.7 nM), (Kuhlman *et al.* 1998) are far below the concentration used in our inhibition screening (25–100 μ M), which raises doubts about the ability of norBup to inhibit P-gp *in vivo*. Thus, the exact consequences of inhibiting P-gp for Bup toxicity in humans remain to be evaluated.

Bup, norBup and Ibo are all significant inhibitors of the efflux of MXR or Ph-a in HEK293-BCRP cells, while Met is not. This confirms that the recovery of P-gp and BCRP substrates is not absolute and justifies systematic BCRP transport studies.

Three other drugs of abuse, D-amphetamine, MDMA and MDA, did not inhibit the transporters tested. Hence, neither P-gp nor BCRP are involved in the transport of D-amphetamine, MDMA or MDA. The polarized transport of MDMA in MDCK and caco-2 cells has never been attributed to P-gp, but to another transport system (Bertelsen *et al.* 2006). We have shown that BCRP should not be considered to be responsible for this transport. Zhu *et al.* (2006b, 2008) showed that P-gp has no apparent effect on the pharmacokinetics and pharmacodynamics of D-amphetamine either *in vivo* or *in vitro*. They found that 100 μ M D-amphetamine does not inhibit the P-gp-mediated transport of Rh-123. Much higher concentrations of D-amphetamine (200 and 500 μ M) were very slightly inhibitory. Inhibition was also detected using doxorubicin (which is also transported by mutant BCRP protein, according to Robey *et al.* 2003) as a functional probe. However they found no P-gp-mediated transport of D-amphetamine in LLC-PK1/MDR1 cells using an uptake assay. This was recently confirmed in a

caco-2 bidirectional study (Crowe & Diep, 2008). Our results also suggest that wild-type BCRP is not involved in D-amphetamine transport. High concentrations of morphine also did not inhibit the transport of either P-gp or BCRP. Human P-gp was reported to poorly transport morphine *in vitro* (Wandel *et al.* 2002), but this has not been confirmed (Feng *et al.* 2008; Schinkel *et al.* 1995). We detected no interaction between morphine and human P-gp or BCRP in our model. Similarly, nicotine did not interact with P-gp in our study, as previously reported (Wang *et al.* 2005). Finally, we find that cocaine did not interact with P-gp, although its polarized transport across bovine brain microvessel endothelial cells suggested that bovine P-gp may transport it (Raje *et al.* 2003).

In conclusion, we have demonstrated the P-gp-mediated transport of norBup, and to a lesser extent of Met, but no involvement of BCRP. However, the clinical consequences of P-gp-mediated Met and norBup transport as well as any P-gp-mediated drug–drug interactions remain to be investigated *in vivo*.

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Statement of Interest

None.

References

- Antoniu T, Tseng AL (2002). Interactions between recreational drugs and antiretroviral agents. *Annals of Pharmacotherapy* **36**, 1598–1613.
- Bertelsen KM, Greenblatt DJ, von Moltke LL (2006). Apparent active transport of MDMA is not mediated by P-glycoprotein: a comparison with MDCK and Caco-2 monolayers. *Biopharmaceutics and Drug Disposition* **27**, 219–227.
- Bonhomme-Faivre L, Benyamina A, Reynaud M, Farinotti R, *et al.* (2008). Disposition of Delta tetrahydrocannabinol in CF1 mice deficient in mdr1a P-glycoprotein. *Addiction Biology* **13**, 295–300.
- Bouer R, Barthe L, Philibert C, Tournaire C, *et al.* (1999). The roles of P-glycoprotein and intracellular metabolism in the intestinal absorption of methadone: *in vitro* studies using the rat everted intestinal sac. *Fundamental & Clinical Pharmacology* **13**, 494–500.

- Crettol S, Deglon JJ, Besson J, Croquette-Krokar M, et al.** (2008). No influence of ABCB1 haplotypes on methadone dosage requirement. *Clinical Pharmacology & Therapeutics* **83**, 668–669; author reply 669–670.
- Crettol S, Digon P, Golay KP, Brawand M, et al.** (2007). In vitro P-glycoprotein-mediated transport of (R)-, (S)-, (R,S)-methadone, LAAM and their main metabolites. *Pharmacology* **80**, 304–311.
- Crowe A, Diep S** (2008). pH dependent efflux of methamphetamine derivatives and their reversal through human Caco-2 cell monolayers. *European Journal of Pharmacology* **592**, 7–12.
- Dagenais C, Graff CL, Pollack GM** (2004). Variable modulation of opioid brain uptake by P-glycoprotein in mice. *Biochemical Pharmacology* **67**, 269–276.
- Dauchy S, Dutheil F, Weaver RJ, Chassoux F, et al.** (2008). ABC transporters, cytochromes P450 and their main transcription factors: expression at the human blood–brain barrier. *Journal of Neurochemistry* **107**, 1518–1528.
- Feng B, Mills JB, Davidson RE, Mireles RJ, et al.** (2008). In vitro P-glycoprotein assays to predict the in vivo interactions of P-glycoprotein with drugs in the central nervous system. *Drug Metabolism and Disposition* **36**, 268–275.
- Hassan HE, Myers AL, Coop A, Eddington ND** (2009). Differential involvement of P-glycoprotein (ABCB1) in permeability, tissue distribution, and antinociceptive activity of methadone, buprenorphine, and diprenorphine: in vitro and in vivo evaluation. *Journal of Pharmaceutical Sciences*. Published online: 15 April 2009. PMID: 19370547.
- Holland ML, Lau DT, Allen JD, Arnold JC** (2007). The multidrug transporter ABCG2 (BCRP) is inhibited by plant-derived cannabinoids. *British Journal of Pharmacology* **152**, 815–824.
- Holland ML, Panetta JA, Hoskins JM, Bebawy M, et al.** (2006). The effects of cannabinoids on P-glycoprotein transport and expression in multidrug resistant cells. *Biochemical Pharmacology* **71**, 1146–1154.
- Hreiche R, Megarbane B, Pirnay S, Borron SW, et al.** (2006). Dexamethasone hepatic induction in rats subsequently treated with high dose buprenorphine does not lead to respiratory depression. *Toxicology and Applied Pharmacology* **217**, 352–362.
- Huang P, Kehner GB, Cowan A, Liu-Chen LY** (2001). Comparison of pharmacological activities of buprenorphine and norbuprenorphine: norbuprenorphine is a potent opioid agonist. *Journal of Pharmacology and Experimental Therapeutics* **297**, 688–695.
- Kadiev E, Patel V, Rad P, Thankachan L, et al.** (2008). Role of pharmacogenetics in variable response to drugs: focus on opioids. *Expert Opinion on Drug Metabolism & Toxicology* **4**, 77–91.
- Kharasch ED, Hoffer C, Whittington D** (2004). The effect of quinidine, used as a probe for the involvement of P-glycoprotein, on the intestinal absorption and pharmacodynamics of methadone. *British Journal of Clinical Pharmacology* **57**, 600–610.
- Kharasch ED, Hoffer C, Whittington D, Sheffels P** (2003). Role of P-glycoprotein in the intestinal absorption and clinical effects of morphine. *Clinical Pharmacology and Therapeutics* **74**, 543–554.
- Kuhlman Jr. JJ, Levine B, Johnson RE, Fudala PJ, et al.** (1998). Relationship of plasma buprenorphine and norbuprenorphine to withdrawal symptoms during dose induction, maintenance and withdrawal from sublingual buprenorphine. *Addiction* **93**, 549–559.
- Levrán O, O'Hara K, Peles E, Li D, et al.** (2008). ABCB1 (MDR1) genetic variants are associated with methadone doses required for effective treatment of heroin dependence. *Human Molecular Genetics* **17**, 2219–2227.
- Mahar Doan KM, Humphreys JE, Webster LO, Wring SA, et al.** (2002). Passive permeability and P-glycoprotein-mediated efflux differentiate central nervous system (CNS) and non-CNS marketed drugs. *Journal of Pharmacology and Experimental Therapeutics* **303**, 1029–1037.
- Martin C, Berridge G, Higgins CF, Mistry P, et al.** (2000). Communication between multiple drug binding sites on P-glycoprotein. *Molecular Pharmacology* **58**, 624–632.
- Maurer HH, Sauer C, Theobald DS** (2006). Toxicokinetics of drugs of abuse: current knowledge of the isoenzymes involved in the human metabolism of tetrahydrocannabinol, cocaine, heroin, morphine, and codeine. *Therapeutic Drug Monitoring* **28**, 447–453.
- Mégarbane B, Marie N, Pirnay S, Borron SW, et al.** (2006a). Buprenorphine is protective against the depressive effects of norbuprenorphine on ventilation. *Toxicology and Applied Pharmacology* **212**, 256–267.
- Mégarbane B, Hreiche R, Pirnay S, Marie N, et al.** (2006b). Does high-dose buprenorphine cause respiratory depression?: possible mechanisms and therapeutic consequences. *Toxicological Reviews* **25**, 79–85.
- Nanovskaya T, Nekhayeva I, Karunaratne N, Audus K, et al.** (2005). Role of P-glycoprotein in transplacental transfer of methadone. *Biochemical Pharmacology* **69**, 1869–1878.
- Nekhayeva IA, Nanovskaya TN, Hankins GD, Ahmed MS** (2006). Role of human placental efflux transporter P-glycoprotein in the transfer of buprenorphine, levo-alpha-acetylmethadol, and paclitaxel. *American Journal of Perinatology* **23**, 423–430.
- Ohlsson A, Lindgren JE, Wahlen A, Agurell S, et al.** (1980). Plasma delta-9 tetrahydrocannabinol concentrations and clinical effects after oral and intravenous administration and smoking. *Clinical Pharmacology and Therapeutics* **28**, 409–416.
- Ohtani M, Kotaki H, Sawada Y, Iga T** (1995). Comparative analysis of buprenorphine- and norbuprenorphine-induced analgesic effects based on pharmacokinetic-pharmacodynamic modeling. *Journal of Pharmacology and Experimental Therapeutics* **272**, 505–510.
- Ortega I, Rodriguez M, Suarez E, Perez-Ruixo JJ, et al.** (2007). Modeling methadone pharmacokinetics in rats in presence of P-glycoprotein inhibitor valsopodar. *Pharmaceutical Research* **24**, 1299–1308.
- Pal D and Mitra AK** (2006). MDR- and CYP3A4-mediated drug–drug interactions. *Journal of Neuroimmune Pharmacology* **1**, 323–339.

- Pirnay S, Herve F, Bouchonnet S, Perrin B, et al.** (2006). Liquid chromatographic-electrospray ionization mass spectrometric quantitative analysis of buprenorphine, norbuprenorphine, nordiazepam and oxazepam in rat plasma. *Journal of Pharmaceutical and Biomedical Analysis* **41**, 1135–1145.
- Polli JW, Wring SA, Humphreys JE, Huang L, et al.** (2001). Rational use of in vitro P-glycoprotein assays in drug discovery. *Journal of Pharmacology and Experimental Therapeutics* **299**, 620–628.
- Raje S, Cao J, Newman AH, Gao H, Eddington ND** (2003). Evaluation of the blood–brain barrier transport, population pharmacokinetics, and brain distribution of buprenorphine analogs and cocaine using in vitro and in vivo techniques. *Journal of Pharmacology and Experimental Therapeutics* **307**, 801–808.
- Robey RW, Honjo Y, van de Laar A, Miyake K, et al.** (2001). A functional assay for detection of the mitoxantrone resistance protein, MXR (ABCG2). *Biochimica et Biophysica Acta* **1512**, 171–182.
- Robey RW, Honjo Y, Morisaki K, Nadjem TA, et al.** (2003). Mutations at amino-acid 482 in the ABCG2 gene affect substrate and antagonist specificity. *British Journal of Cancer* **89**, 1971–1978.
- Robey RW, Steadman K, Polgar O, Morisaki K, et al.** (2004). Pheophorbide is a specific probe for ABCG2 function and inhibition. *Cancer Research* **64**, 1242–1246.
- Rodriguez M, Ortega I, Soengas I, Suarez E, et al.** (2004). Effect of P-glycoprotein inhibition on methadone analgesia and brain distribution in the rat. *Journal of Pharmacy and Pharmacology* **56**, 367–374.
- Sadeque AJ, Wandel C, He H, Shah S, et al.** (2000). Increased drug delivery to the brain by P-glycoprotein inhibition. *Clinical Pharmacology and Therapeutics* **68**, 231–237.
- Scherrmann JM** (2005). Expression and function of multidrug resistance transporters at the blood–brain barriers. *Expert Opinion on Drug Metabolism and Toxicology* **1**, 233–246.
- Schinkel AH, Wagenaar E, van Deemter L, Mol CA, et al.** (1995). Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *Journal of Clinical Investigation* **96**, 1698–1705.
- Störmer E, Perloff MD, von Moltke LL, Greenblatt DJ** (2001). Methadone inhibits rhodamine123 transport in Caco-2 cells. *Drug Metabolism and Disposition* **29**, 954–956.
- Suzuki T, Zaima C, Moriki Y, Fukami T, et al.** (2007). P-glycoprotein mediates brain-to-blood efflux transport of buprenorphine across the blood–brain barrier. *Journal of Drug Targeting* **15**, 67–74.
- Takeuchi T, Yoshitomi S, Higuchi T, Ikemoto K, et al.** (2006). Establishment and characterization of the transformants stably-expressing MDR1 derived from various animal species in LLC-PK1. *Pharmaceutical Research* **23**, 1460–1472.
- Taub ME, Podila L, Ely D, Almeida I** (2005). Functional assessment of multiple P-glycoprotein (P-gp) probe substrates, influence of cell line and modulator concentration on P-gp activity. *Drug Metabolism and Disposition* **33**, 1679–1687.
- Walsh SL, Preston KL, Stitzer ML, Cone EJ, Bigelow GE** (1994). Clinical pharmacology of buprenorphine: ceiling effects at high doses. *Clinical Pharmacology and Therapeutics* **55**, 569–580.
- Wandel C, Kim R, Wood M, Wood A** (2002). Interaction of morphine, fentanyl, sufentanil, alfentanil, and loperamide with the efflux drug transporter P-glycoprotein. *Anesthesiology* **96**, 913–920.
- Wang JS, Markowitz JS, Donovan JL, Devane CL** (2005). P-glycoprotein does not actively transport nicotine and cotinine. *Addiction Biology* **10**, 127–129.
- Wang JS, Ruan Y, Taylor RM, Donovan JL, et al.** (2004). Brain penetration of methadone (R)- and (S)-enantiomers is greatly increased by P-glycoprotein deficiency in the blood–brain barrier of Abcb1a gene knockout mice. *Psychopharmacology* **173**, 132–138.
- Yassen A, Kan J, Olofson E, Suidgeest E, et al.** (2007). Pharmacokinetic-pharmacodynamic modeling of the respiratory depressant effect of norbuprenorphine in rats. *Journal of Pharmacology and Experimental Therapeutics* **321**, 598–607.
- Zhu HJ, Wang JS, Markowitz JS, Donovan JL, et al.** (2006a). Characterization of P-glycoprotein inhibition by major cannabinoids from marijuana. *Journal of Pharmacology and Experimental Therapeutics* **317**, 850–857.
- Zhu HJ, Wang JS, DeVane CL, Williard RL, et al.** (2006b). The role of the polymorphic efflux transporter P-glycoprotein on the brain accumulation of d-methylphenidate and d-amphetamine. *Drug Metabolism and Disposition* **34**, 1116–1121.
- Zhu HJ, Wang JS, Donovan JL, Jiang Y, et al.** (2008). Interactions of attention-deficit/hyperactivity disorder therapeutic agents with the efflux transporter P-glycoprotein. *European Journal of Pharmacology* **578**, 148–158.