

ORIGINAL
ARTICLE

P-glycoprotein is not involved in the differential oral potency of naloxone and naltrexone

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P-glycoproteinReceived 14 November 2008;
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jules.desmeules@hcuge.ch**ABSTRACT**

The poor oral bioavailability of the opioid receptor antagonist naloxone (NA) when compared with naltrexone (NX) may be related to a greater interaction of naloxone with the efflux drug transporter P-glycoprotein (P-gp). We studied the involvement of P-gp in the transepithelial transport of the two opioid receptor antagonists, using a validated human in vitro Caco-2 cell monolayer model. The bidirectional transport of NA and NX (1, 50 and 100 μM) across the monolayers was investigated in the presence and absence of the specific P-gp inhibitor GF120918 (4 μM). NA and NX showed equal transport rates between the apical-to-basolateral (A–B) and the basolateral-to-apical (B–A) directions and neither the influx nor the efflux transport was affected by the P-gp inhibitor ($P > 0.05$). In conclusion, NA and NX are not P-gp substrates. The differential oral bioavailability of the two opioid antagonists is P-gp independent.

INTRODUCTION

Naloxone (NA), a synthetic N-allyl derivative of oxycodone, and its analogue naltrexone (NX) are both opioid antagonists at the mu, kappa and sigma opioid receptor sites [1] (Figure 1). Naloxone is the treatment of choice for opioid overdose, regardless of the type of opioid, as it permits a dose dependant reversal of opioid adverse events in the central nervous system. It is mostly administered by intravenous injection for complete bioavailability and rapid onset of action. Naloxone is mainly metabolized in the liver by glucuroconjugation into naloxone-3-glucuronide [2] and eliminated by the renal route [3]. Naltrexone is approximately twice as potent as naloxone [4] and is mostly used orally for the treatment of alcohol dependence. Naltrexone is metabolized by dihydrodiol dehydrogenase to the main active metabolite 6-beta-naltrexol, via extensive hepatic and extra-hepatic routes [5]. Although a weaker opioid antagonist than naltrexone, 6-beta-naltrexol possibly contributes to the long duration of action observed with the parent compound, particularly because plasma

concentrations of the metabolite are higher than those of the parent drug. Naltrexone and 6-beta-naltrexol are mainly excreted by the renal route [6].

Naloxone has a low oral bioavailability when compared to naltrexone [7–10]. It is, however, unclear whether the very limited systemic availability of oral naloxone is due to a more extensive first pass metabolism, when compared with naltrexone, or to a poor gastrointestinal absorption. P-glycoprotein (P-gp) (ABCB1/MDR1) is expressed in the major organs associated with drug absorption, distribution and elimination, therefore playing a major role in pharmacokinetics of several drugs.

MATERIALS AND METHODS**Materials**

Caco-2 cells (TC7 clone) were kindly provided by Tea Fevr, PhD (ISREC, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland). Penicillin–streptomycin was purchased from Sigma-Aldrich GmbH (Steinheim, Germany) and all other cell culture reagents

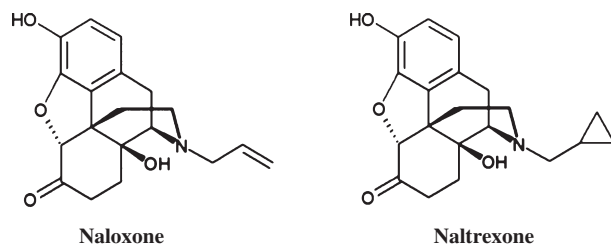


Figure 1 Chemical structures of naloxone and naltrexone.

from Gibco BRL (Paisley, Scotland). Naloxone and naltrexone were purchased from Sigma-Aldrich GmbH and GF120918 was kindly provided by GlaxoSmithKline (Middlesex, UK).

Cell culture

Caco-2 cells (TC7 clone) were used at passages 21–25. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM Glutamax, Gibco BRL) supplemented by 20% fetal bovine serum (FBS, Gibco BRL), 1% nonessential amino acids (NEA, Gibco BRL), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich), at 37 °C in a humidified atmosphere with 5% CO₂. At 85–95% confluency, Caco-2 cells were treated with 0.25% trypsin–EDTA (Gibco BRL) and seeded at a density of 65 000 cells/cm² on polycarbonate membranes of Transwells (12-mm diameter, 1.13 cm², 0.4 µm pore size, 12-well plates; Costar, Cambridge, MA, USA), previously equilibrated for 1 h in the culture medium. Medium was changed the day after seeding and every other day thereafter [apical volume (A): 0.5 mL, basolateral volume (B): 1.5 mL]. Monolayers were used for transport studies 20–21 days post seeding to allow full maturation of the cells, including P-gp expression and appropriate tight junctions.

Measurement of transepithelial electrical resistance

Transepithelial electrical resistance (TEER) was checked every 5 days during the 21-day maturation of the monolayers. Prior to bidirectional transport studies, medium was removed from both apical and basolateral chambers and monolayers were rinsed three times with the transport buffer Hank's balanced salt solution (HBSS), supplemented with 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethane-sulfonic acid (HEPES) (Gibco BRL) and pH adjusted to 7.4 with 0.5 M NaOH. Cells were equilibrated in the same buffer for 1 h and the integrity of each monolayer was checked by measuring

its TEER with a Millicell-ERS ohmmeter (Millipore Corp., Bedford, MA, USA). Resistance was also checked immediately after the experiments.

Transmission electron microscopy and Western blotting of P-glycoprotein

Transmission electron microscopy and Western blotting of P-glycoprotein were performed in the context of the Caco-2 model validation in our previous study (see [11]).

Transport studies

After measurement of TEERs, HBSS buffer was removed from each chamber. Apical to basolateral (A–B) transport was initiated by replacing basolateral (B) buffer with 1.5 mL of fresh HBSS supplemented with 25 mM HEPES, 1% DMSO and pH adjusted to 7.4 with NaOH 0.5 M, and replacing apical (A) buffer with 0.5 mL of the drug solution in the same buffer (HBSS/HEPES pH 7.4). In other wells, B–A transport was initiated by replacing (A) buffer with 0.5 mL of fresh HBSS/HEPES pH 7.4 and (B) buffer with the drug solution in the same buffer (1.5 mL). For the P-gp inhibition studies, GF120918 (4 µM) was present in both chambers. Samples (100 µL) were removed from each receiver chamber at various times (30, 60, 90, 120 and 180 min) and replaced with buffer to maintain constant volumes. The 3-h transport studies were performed at a constant agitation rate (50 rpm) using a circular shaker (type SSM1, Stuart®) in an incubator (37 °C, 5% CO₂ and humidified atmosphere). After the transport studies, all aliquots were stored at –20 °C until analysis.

Analytical method

Naloxone and naltrexone analysis for the 50 and 100 µM assays was performed by LC-UV at 230 nm. Separation was carried out on a Zorbax eclipse XDB-C8 (150 × 4.6 mm i.d., particle size: 5 µm) from Agilent, coupled with a guard column with the same stationary phase. The mobile phase consisted of a mixture of acetonitrile and orthophosphoric acid (50 mM) adjusted to pH 4.2 with sodium hydroxide 4N and was delivered at 0.9 mL/min. A gradient elution was used in which the mobile phase composition was changed from 12–88% to 30–70% (ACN-Orthophosphoric acid) within 5 min, maintained at 30–70% until $t = 8$ min, and returned to the initial composition from $t = 8$ to $t = 9$ min. Naloxone and naltrexone analysis for 1 and 10 µM assays was achieved using an LC-MS system (Esquire 3000 + Ion-Trap) from Bruker daltonics (Billerica, MA, USA) equipped with an

electrospray source working in positive ion mode. The ion transitions monitored in multiple reaction-monitoring modes were m/z 342 \rightarrow 324, and 328 \rightarrow 310 for naltrexone and naloxone, respectively. Optimized ESI source voltages were as follows: spray needle at +4.3 kV, end plate offset at -500 V, capillary exit offset at -200 V, skimmer 1 at -107.4 V. Further ion source parameters were 70 psi nebulizer gas and 11 L/min of drying gas with a temperature of 350 °C. Separation was achieved with an XTerra[®] MS C18 column (100 mm \times 2.1 mm i.d., particle size: 3.5 μ m) from Waters (Milford, MA, USA) at 0.3 mL/min. Mobile phase consisted of ammonium formate 20 mM and acetonitrile (20–80%).

In all cases, the standard curves were obtained by linear regression of measured peak area vs. concentration and used to calculate concentrations of the analytes in unknown and QC samples. Samples consisted of aliquots removed from the receiver chambers (drug solution in the aqueous buffer HBSS). No additional treatment was needed. No extraction was required and the samples were directly injected into the HPLC system without need for an internal standard. The performance of both methods, in terms of reproducibility, repeatability and linearity were assessed before analysis (data not shown).

Calculations

TEER was calculated from the following equation [12]:

$$\text{TEER} = (\text{TEER}_{\text{mono}} - \text{TEER}_{\text{blank}}) \times A$$

where $\text{TEER}_{\text{mono}}$ is the cell monolayer and polycarbonate porous membrane resistance, $\text{TEER}_{\text{blank}}$ the polycarbonate porous membrane resistance and A the polycarbonate porous membrane surface area (1.13 cm²).

Apical to basolateral ($P_{\text{app}}(\text{A-B})$) and basolateral to apical ($P_{\text{app}}(\text{B-A})$) apparent permeability coefficients were calculated according to Artursson [13] using the following equation:

$$P_{\text{app}}(\text{cm/s}) = (dQ/dt)/(A \times C_0 \times 60)$$

where dQ/dt (μ g/min) is the permeability rate of the drug, calculated from the regression line of the time points of sampling, A is the surface area of the monolayer (cm²) and C_0 the initial drug concentration in the donor chamber (μ g/L).

Karlsson et al. [14] suggested that there is involvement of a drug efflux transporter in Caco-2 cells if the efflux ratio ($\text{TR} = P_{\text{app}}(\text{B-A})/P_{\text{app}}(\text{A-B})$) is >2 and if a

decreased secretory transport rate ($P_{\text{app}}(\text{B-A})$) is observed in the presence of an inhibitor of the transporter in question. For a compound with an efflux ratio of 1.5–2.0, a positive effect of the inhibitor confirms the implication of the efflux transporter [15].

Statistics

The unpaired bilateral Student's t test was used for statistical comparison of the transport rates in each direction and the transport rates in the presence or absence of the P-gp inhibitor for a particular direction (XLstat version 5.0; Addinsoft, New York, NY, USA). A P value of <0.05 was considered significant.

RESULTS

Differentiation and integrity of Caco-2 cell monolayers

Transmission electron microscopy (TEM)

Histological examination showed a continuous differentiated cell monolayer presenting microvilli on the apical cell surface, interdigitations, numerous desmosomes, and tight junctions (see [11]).

Transepithelial electrical resistance

Caco-2 cell monolayers with TEER values between 300 and 400 Ω .cm² were used in the study. Measurements conducted after the experiments displayed similar values and confirmed the integrity of the monolayers during all of the experiments. No tendency towards an effect on TEER was observed under the various experimental conditions (pH, substrates and inhibitors).

P-glycoprotein expression and activity

Immunoblotting and transepithelial transport of P-gp probe

The Western blot analysis revealed a C219 antibody-reactive band of 170 kDa corresponding to P-gp expression (see [11]). The P-gp probe rhodamine 123 (5 μ M) [16] showed a positive interaction with P-glycoprotein in our Caco-2 cell monolayers, with a secretory transport markedly inhibited by GF120918 (4 μ M) ($P_{\text{app}}(\text{B-A})$: $7.1 \pm 0.5 \cdot 10^{-6}$ vs. $1.3 \pm 1.4 \cdot 10^{-6}$, $P < 0.05$).

Transepithelial transport of naloxone and naltrexone

The bidirectional transport of naloxone and naltrexone (1, 50 and 100 μ M) was investigated at pH 7.4/7.4 in the presence and absence of 4 μ M GF120918. The lowest naloxone and naltrexone concentrations tested (1 μ M) correspond, respectively, to six times the plasma

concentration measured 2 min after an injection of 0.4 mg naloxone (0.01 µg/mL) [17] and 45 times the peak plasma concentration ($C_{max} = 13.7$ ng/mL) measured after multiple oral doses of 50mg naltrexone [18].

Naloxone and naltrexone showed no statistical difference between apical-to-basolateral ($P_{app}(A-B)$) and basolateral-to-apical ($P_{app}(B-A)$) transport. The efflux ratio ($TR = P_{app}(B-A)/P_{app}(A-B)$) of the two molecules was between 1.1 and 1.3, indicating that naloxone and naltrexone are not actively transported by an efflux transporter. This was confirmed by the use of the P-gp inhibitor, GF120918, which failed to alter the observed ratio (Table I, Figure 2).

DISCUSSION

The results of our study clearly show that neither naloxone nor naltrexone are P-gp substrates. Indeed, no significant efflux ratio was observed for the two molecules across the P-gp-overexpressing Caco-2 cell monolayers, as their respective fluxes in the absorptive and secretory directions were equivalent at all concentrations tested. Moreover, a potent P-gp inhibitor, GF120918, had no effect on bidirectional flux, either by the inhibition of efflux transport or by the enhancement of absorptive flux ($P > 0.05$). The absorptive apparent permeability coefficients of naloxone and naltrexone ($P_{apps} > 10 \times 10^{-6}$) show otherwise that the two opioid

Table I Apparent permeability coefficients P_{app} (cm/s) of naloxone and naltrexone in the presence and absence of GF120918 at pH 7.4/7.4.

Drug concentration ± P-gp inhibitor	P_{app} (A-B) (cm/s) (10^{-6}) pH 7.4/7.4	P_{app} (B-A) (cm/s) (10^{-6}) pH 7.4/7.4	Efflux ratio (TR)
Naloxone			
1 µM	25.4 ± 0.8	29.2 ± 1.6	1.1 (no net efflux)
+ 4 µM GF120918	22.2 ± 2.6	30.7 ± 2.0	1.3
50 µM	28.0 ± 0.5	34.1 ± 0.5	1.2 (no net efflux)
+ 4 µM GF120918	28.7 ± 1.3	33.8 ± 2.9	1.1
100 µM	28.4 ± 0.3	31.4 ± 2.1	1.1 (no net efflux)
+ 4 µM GF120918	26.6 ± 1.4	33.0 ± 1.8	1.2
Naltrexone			
1 µM	15.1 ± 0.6	19.7 ± 0.5	1.3 (no net efflux)
+ 4 µM GF120918	13.7 ± 0.8	19.9 ± 0.9	1.4
50 µM	14.6 ± 0.2	20.0 ± 1.1	1.3 (no net efflux)
+ 4 µM GF120918	14.7 ± 0.3	20.1 ± 1.0	1.3
100 µM	13.9 ± 0.4	17.5 ± 0.6	1.2 (no net efflux)
+ 4 µM GF120918	19.8 ± 5.2	17.7 ± 0.2	0.8

Values are the mean ± SD of three experiments. $P < 0.05$.

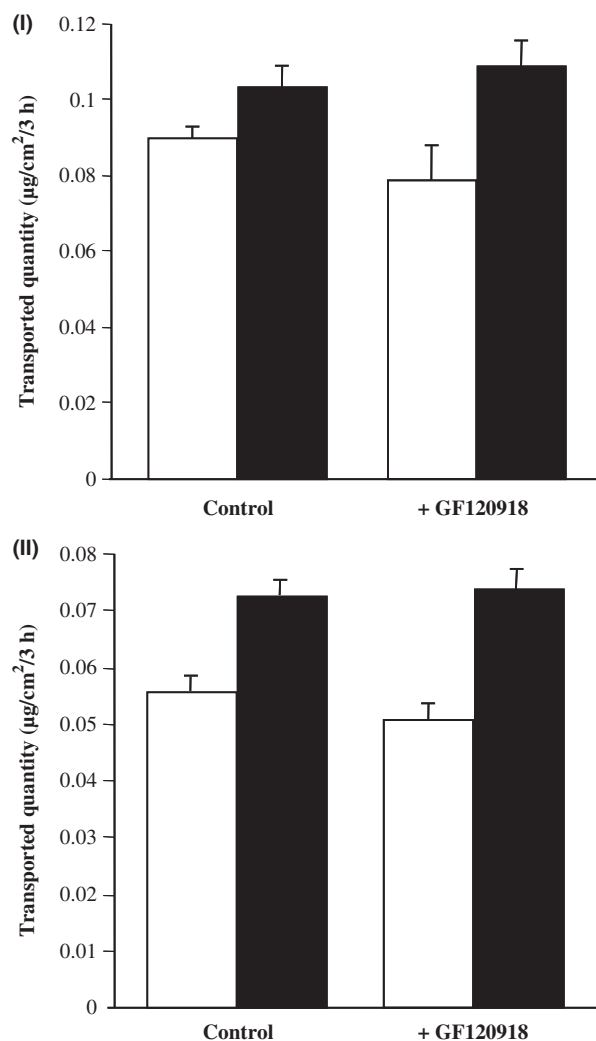


Figure 2 Transepithelial transport of naloxone (I) and naltrexone (II) across Caco-2 cell monolayers in the presence and absence of GF120918.

Naloxone (I) and naltrexone (II) (1 µM) were added to the apical side (open columns) or the basolateral side (solid columns) of the monolayers in the presence (+GF120918) or absence (control) of 4 µM of GF120918 at pH 7.4/7.4. Data are mean ± SD of three experiments. $P < 0.05$.

antagonists are well absorbed compounds [19]. Our transepithelial bidirectional study indicates that passive diffusion seems to be the major mechanism of naloxone and naltrexone transmembrane transit.

Experimental animal data suggest that naloxone may interact with P-glycoprotein. Naloxone has been shown to stimulate ATPase activity in the plasma membranes of the multidrug-resistant Chinese hamster ovary (CHO) cell line CR1R12, as well as in purified reconstituted P-gp liposomes, although only at very high concentrations

(>100 μM) [20]. Our assays at lower concentrations (1 and 50 μM) do not confirm these results. Naloxone also weakly inhibited vinblastine binding to the plasma membranes of multidrug-resistant CHO (B30) cells at high concentrations (100 μM) [21]. In another experimental study in P-gp expressing CHO cells [22], naloxone caused a decrease in P-glycoprotein phosphorylation. Naloxone seems indeed to interact with animal P-gp, but with very low affinity, mainly resulting in weak inhibition of the efflux transporter. No data concerning the interaction of naltrexone with P-glycoprotein or any other "ATP-binding cassette" (ABC) or "solute-linked carriers" (SLC) transporter are available in the literature.

Naloxone and naltrexone are two structurally related opioid antagonists, mainly metabolized in the liver into naloxone-3-glucuronide and 6-beta-naltrexol, respectively [2,5]. Naloxone and naltrexone show similar potencies when administered parenterally. However, after oral administration, naltrexone is thought to be significantly more potent and long-lasting than naloxone in antagonizing the central effects of opioids [23]. Indeed, after oral administration, naloxone bioavailability is less than 2% [7,8,24,25], whereas naltrexone is between 5 and 40% [9,10]. The huge difference observed in bioavailability after oral administration renders oral naloxone useless in opioid and alcohol addiction treatment. It has, however, led to the development and commercialization of combinations of naloxone and opioid agonists, such as pentazocine and buprenorphine, to avoid the illicit parenteral use of the opioid agonist [26,27], as well as oxycodone to prevent peripherally mediated constipation without interfering with the central analgesic effect [28].

Naloxone and naltrexone show very similar liposolubility (LogPs: 1.5 and 1.4, respectively), however, naltrexone has a higher pKa than naloxone (8.4 vs. 7.9) [29,30]. Hence, at gastrointestinal pH conditions (pH 5–8) [31] as well as at physiological pH, the percentage of the ionized form of naltrexone is greater than that of naloxone. Consequently, the physicochemical properties of the two opioid antagonists do not favour a greater gastrointestinal permeability of naltrexone as compared to naloxone. Additionally, our experimental study showed an almost twofold greater naloxone permeability across Caco-2 cells compared to naltrexone (ex, at 1 μM ; $P_{\text{app}}(\text{A-B})$: $25.4 \pm 0.8 \cdot 10^{-6}$ vs. $15.1 \pm 0.6 \cdot 10^{-6}$, $P < 0.05$).

Evidence largely implicates hepatic first-pass metabolism in the poor oral bioavailability of naloxone, based on studies investigating the systemic biodisposition of

the drug after oral and intravenous administration [7,8,24,25,32]. However, other mechanisms such as P-glycoprotein or other efflux drug transporters may be involved in limiting its gastrointestinal absorption and thus oral bioavailability. Our results show that the poor oral bioavailability of naloxone is not linked to a limited gastrointestinal absorption of the drug.

In conclusion, our study excludes the involvement of P-glycoprotein in the poor oral bioavailability of naloxone as compared to naltrexone. Naloxone gastrointestinal absorption does not seem to be the major cause of its poor oral bioavailability. In fact, rapid and extensive metabolism of the opioid antagonist appears as the more plausible factor to be considered.

CONFLICTS OF INTEREST

The authors state no conflict of interest.

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