# Lack of Interaction of the NMDA Receptor Antagonists Dextromethorphan and Dextrorphan with P-Glycoprotein

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**Abstract:** The anti-N-methyl-D-aspartate (NMDA) effect of dextromethorphan (DEM) seems to be mainly related to the unchanged drug rather than to its more potent metabolite dextrorphan (DOR). The aim of our study was to assess the involvement of P-glycoprotein (P-gp) and pH conditions in the transmembranal transport of these two NMDA antagonists, using a human *in vitro* Caco-2 cell monolayer model. Transmission electron microscopy, transepithelial electrical resistance, [$^3$H]-mannitol permeability, Western blot analysis and the bidirectional transport of the positive controls, rhodamine and digoxine were used to confirm model's integrity and validity. The bidirectional transport of DEM and DOR (1 to 100µM) across the monolayers was investigated in the presence and absence of the P-gp inhibitor cyclosporine A (10µM) at two pH conditions (pH 6.8/7.7-pH 7.4/7.4) and assessed with the specific and more potent P-gp inhibitor GF120918 (4µM). Analytical quantification was achieved using high performance liquid chromatography. At a pH gradient, DEM and DOR were subject to a significant active efflux transport (Papp(B-A) > 2-3x Papp(A-B); $p<0.01$). However, neither the influx nor the efflux was affected by P-gp inhibitors. At physiological pH, we observed no more efflux of the drugs and no influence of the inhibitors.

In conclusion, dextromethorphan and dextrorphan are not P-gp substrates. However, pH-mediated efflux mechanisms seem to be involved in limiting DEM gastrointestinal absorption. The preferential anti-NMDA central effect of DEM appears to be P-gp independent.

**Keywords:** P-glycoprotein, Caco-2 cells, Transport, Dextromethorphan, Dextrorphan.

## 1. INTRODUCTION

Dextromethorphan (DEM), a non-narcotic morphinan derivative devoid of opioid activity, is widely used as an antitussive agent. Due to its *in vivo* functional noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist properties, DEM showed antinociceptive, anticonvulsant, neuromodulatory and neuroprotective properties at high doses in several clinical trials [1-4]. DEM is metabolized to dextrorphan (DOR) by cytochrome P450 (CYP) 2D6 and to a lesser extend to 3-methoxymorphinan (3-MM) by CYP3A4 (Fig. (**1**)) [5]. *In vitro* studies showed that DOR was a more potent NMDA antagonist than the unchanged DEM [6-8]. However, experimental and clinical evidence suggest that the antinociceptive, neuromodulatory and neuroprotective *in vivo* effect of DEM result mainly from a central action of unchanged DEM rather than from its more active metabolite DOR [1,9].

P-glycoprotein (P-gp/MDR1/ABCB1) is a 170kD cell-membrane protein classified as a member of the ATP-binding cassette superfamily of transport proteins (ABC transporters). It is localized in a number of tissue compartments, including the luminal membrane of intestinal enterocytes, the canalicular membrane of hepatocytes, the luminal membrane of renal proximal tubular cells and the luminal membranes of brain capillary endothelial cells [10,11]. P-gp is a protective physiological barrier that limits substrate xenobiotic exposure in the body playing therefore a crucial role in drug pharmacokinetics [12]. P-gp mediated efflux of a substrate drug, among other pharmacokinetic effects, limits its intestinal absorption and blood-brain-barrier penetration. It is a broad-spectrum multidrug resistance glycoprotein and many structurally different drugs have been recognized as substrates (digoxine, fexofenadine) [13,14], inhibitors (cyclosporine, GF 120918) [15-17] or inducers (rifampicine) of P-gp [18].

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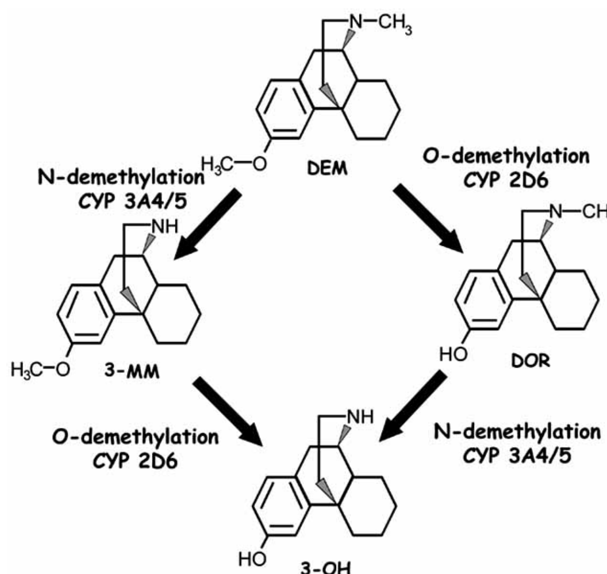


**Fig. (1).** Metabolism pathways of dextromethorphan.
DEM: dextromethorphan, DOR: dextrorphan, 3-MM: 3-methoxymorphinan, 3-OH: 3-hydroxymorphinan.

Since drug biodisposition can be modulated by P-glycoprotein, the degree of its interaction with dextromethorphan and/or dextrorphan may be of importance for achieving the expected neuromodulatory pharmacodynamic effect.

The present study assessed if a stronger interaction of DOR with the efflux drug transporter P-gp could explain the better DEM *in vivo* neuromodulatory activity.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Caco-2 cells (TC7 clone) were kindly provided by Martin Rumbo, PhD (ISREC, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland). MES was purchased from Ap-

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pliChem GmbH, penicillin-streptomycin from Sigma Aldrich GmbH and all other cell culture reagents from Gibco BRL. [$^3$H]-mannitol was purchased from Perkin Elmer Life Sciences. Dextromethorphan, rhodamine and digoxine were purchased from Sigma Aldrich GmbH, dextrorphan from Roche Pharma (Switzerland), cyclosporine A from Novartis Pharma (Switzerland) and GF120918 was kindly provided by GlaxoSmithKline (GSK, United Kingdom).

### 2.2. Cell Culture

Caco-2 cells (TC7 clone) were used at passages 40 to 55. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM Glutamax, Gibco BRL) supplemented by 10% fetal bovine serum (FBS, Gibco BRL), 1% nonessential amino acids (NEA, Gibco BRL), 100U/ml penicillin and 100µg/ml streptomycin (Sigma Aldrich) at 37°C in a humidified atmosphere with 5%$CO_2$ [19]. At 80-90% confluency, Caco-2 cells were treated with 0.25% trypsine-EDTA (Gibco BRL) and seeded at a density of 65.000cells/cm$^2$ on polycarbonate membranes of Transwells (12mm diameter, 1.13cm$^2$, 0.4µm pore size, 12 well plates; Costar, Cambridge, MA), previously equilibrated during one hour. Medium was changed the day after seeding and every other day thereafter (apical volume (A): 0.5ml, basolateral volume (B): 1.5ml) and monolayers were used for transport studies 20-21 days post-seeding to allow full maturation of the cells, including P-gp expression and appropriate transepithelial electrical resistance [20,21].

### 2.3. Measurement of TEER

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 25mM N-(2-hydroxylethyl) piperazine-N'-2ethane-sulfonic acid (HEPES) (Gibco-BRL) and pH-adjusted to 7.4 with NaOH 0.5M. They were equilibrated in the same buffer for one hour and the integrity of each monolayer was checked by measuring its transepithelial electrical resistance (TEER) with a Millicell-ERS ohmmeter (Millipore Corp., Bedfort, MA). Resistance was also checked immediately after the experiments.

### 2.4. Transmission Electron Microscopy

Caco-2 monolayers on polycarbonate membranes were fixed in 2% glutaraldehyde (phosphate buffer, pH 7.4), cutt in three transversely, post-fixed in 1% osmium tetroxyde, contrasted "in bloc" with uranyl acetate, dehydrated through graded ethanols and embedded in Epon 812. Thin gold sections were stained with uranyl acetate and lead citrate and finally examined with a Phillips 400 electron microscope.

### 2.5. Electrophoresis and Western Blotting of P-Glycoprotein

Caco-2 monolayers were washed twice with cold PBS then gently collected in an Eppendorf. The cells were centrifuged in 0.5 ml of PBS (700 tours/minute for 5 minutes). The pellet was resuspended and lysed in 120µl of a buffer containing 1% Nonidet P-40, 1% sodium cholate, 0.05% SDS, 20 mM Tris (pH 7.5) and 100 mM NaCl. Half of the lysate was then sonicated on ice to maximize protein release. Protein concentration was determined using the Bradford dye-binding assay (Bio-Rad), according to the manufacturer's protocol, with BSA as the standard. Ten micrograms of total protein was separated by SDS/PAGE (10%) under reducing conditions and blotted onto pure nitrocellulose membranes (Bio-rad). Membranes were incubated with anti-human MDR1 antibody (1:100, mouse monoclonal antibody to P-glycoprotein (C219), ALEXIS Biochemicals), followed by a horseradish peroxidase-conjugated secondary antibody (1:100, Bio-Rad). The immunoreactive proteins were detected with an enhanced chemiluminescence kit (ECL, Amersham Pharmacia) according to the manufacturer's protocol, by using ECL-Hyperfilm (Amersham Pharmacia).

### 2.6. Paracellular Transport of [$^3$H]-mannitol

Apical to basolateral permeability (A-B) of [$^3$H]-mannitol a radiolabeled paracellular marker (activity: 0.5 µCi/ml), was measured over 3 hours to monitor the integrity of the tight junctions of the monolayers. Samples taken from the basolateral side (100µl) at various times were counted in a Pico-Fluor 15 medium (Perkin Elmer Life Sciences) using a liquid scintillation counter (Packard Instruments).

### 2.7. Transport Studies

pH 6.8/7.4 conditions: 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 1.5ml of fresh HBSS supplemented with 25mM HEPES and pH-adjusted to 7.4 (1.5ml) and apical (A) buffer with the drug solution in HBSS supplemented with 10mM MES (AppliChem GmbH), pH-adjusted to 6.8 with 0.5M NaOH (0.5ml). B-A transport was initiated by replacing (A) buffer with 0.5ml of fresh HBSS/MES pH 6.8 and (B) buffer with the drug solution in HBSS/HEPES (1.5ml). For the P-gp inhibition studies, the inhibitor was present in both chambers. Cyclosporine A and GF120918 were used respectively at 10 and 4µM [22,23]. Samples (100µl) were removed from each receiver chamber at various times (30, 60, 90, 120 and 180min) and replaced with buffer to maintain constant volumes. The three hour transport studies were performed at a constant agitation rate (50rpm) using a circular shaker (type SSM1, Stuart®) in an incubator (37°C, 5% $CO_2$ and humidified atmosphere) [24].

pH 7.4 /7.4 conditions: As described for the pH 6.8/7.4 conditions except that (A) and (B) buffers and drug solutions were made with HBSS supplemented with 25mM HEPES pH 7.4.

After the transport study, all aliquots were stored at –20°C until analysis.

### 2.8. Analytical Method

DEM, DOR and rhodamine analysis was performed using liquid chromatography coupled to the fluorescence detector. For DEM and DOR determination, MN phenyl column was used. The mobile phase consisted of a mixture of acetonitrile and orthophosphoric acid 50 mM (18/82) pH 3.0 and was delivered at 0.8 ml/min. For rhodamine determination, a Zorbax Eclipse XDB-C8 column was used. The mobile phase consisted of a mixture of acetonitrile and orthophosphoric acid 50 mM (30/70) pH 3.0 and was delivered at 0.9 ml/min. Fluorescence was measured with emission and excitation wavelengths set at 280 nm and 310 nm respectively for DEM and DOR and 500 nm and 525 nm for rhodamine. In all cases, 50 µl or 30µl (for 1-10µM and 50-100µM respectively) were directly injected into the (HPLC) system. Digoxine analysis was performed by an Agilent HP1100 liquid chromatography coupled to an ion trap mass spectrometer (Esquire 3000+, Bruker daltonics) equipped with an electrospray source working in positive ion mode. The ion count cumulative target for the ion trap mass analyzer was 10.000, with a maximum accumulation time of 200 ms. 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. Mass spectra were recorded in the full scan mode with a mass range from $m/z = 100$ to 1000 and the mass 803.4 corresponding to the $[M+Na]^+$ adduct ion of digoxine was extracted for quantitation. Separation was achieved with an XTerra® MS C18 column (100mm×2.1mm i.d., 3.5 µm, Waters, Ireland) at 0.3 ml/min using a gradient of ammonium acetate 10mM (solvent A) and acetonitrile (solvent B), programmed as follows: 0 min 20% B, 0–3 min from 20% B to 80% B, 3-3.5 min from 80% B to 20% B and post-run 5 min with 20% B. Injection volume was 20 µl. The standard curves were obtained by weighted least-squares regression (weighting=1/x$^2$) of the measured peak area versus the analyte concentra-

tions. The standard curves were then used to calculate concentrations of the analytes in unknown and QC samples. In all cases, the samples consisted of the 100µl 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.

### 2.9. Calculations and Statistics

- TEER was calculated from the following equation [25]:

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

Where the $TEER_{mono}$ is the cell monolayer and polycarbonate porous membrane resistance, $TEER_{blank}$ is the polycarbonate porous membrane resistance and A the polycarbonate porous membrane surface area (1.13 $cm^2$).

- Apical to basolateral ($P_{app}$ (A-B)) and basolateral to apical ($P_{app}$ (B-A)) apparent permeability coefficients were calculated according to Artursson (1990) [26] using the following equation:

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

Where dQ/dt (µg/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 ($cm^3$) and $C_0$ is the initial drug concentration in the donor chamber (µg/l).

Karlsson *et al.* [27] suggested an involvement of a drug efflux transporter in the investigated Caco-2 cells if the efflux ratio (TR = $P_{app}$(B-A)/$P_{app}$(A-B)) is > 2 and if an increased absorptive rate ($P_{app}$(A-B)) and a decreased secretory transport rate ($P_{app}$(B-A)) is observed in the presence of an inhibitor of this transporter.

- Microsoft Excel was used for calculating $P_{apps}$. Unpaired Student test was used for statistical comparison of the transport rates in each direction, the transport rate in the presence and absence of an inhibitor for a particular direction and the transport efflux ratios in relation to pH conditions (Xlstat version 5.0). A $p<0.01$ was considered significant.

## 3. RESULTS

### 3.1. Differenciation and Integrity of Caco-2 cell Monolayers

#### *3.1.1. Transmission Electron Microscopy (TEM)*

Histological examination showed a continuous differentiated cell monolayer presenting microvillus on the apical cell surface, interdigitations, numerous desmosomes (maculae adherents) and tight junctions (Fig. (**2**)).

#### *3.1.2 Transepithelial Electrical Resistance (TEER)*

Caco-2 cell monolayers with TEER values comprised between 250 and 450 ($\Omega.cm^2$) were commonly used in the study. Measurements conducted after the experiments displayed similar values and confirmed the integrity of the monolayer during all the experiments. No tendency towards an effect on TEER of the various experiment conditions (pH, substrates, inhibitors) was observed.

#### *3.1.3. Paracellular Transport of [$^3$H]-mannitol*

The transport rate of radiolabeled [$^3$H]-mannitol was less than 1% per hour showing functional tight junctions. As for TEER, no tendency towards an effect of the various experiment conditions (pH, substrates, inhibitors) on [$^3$H]-mannitol permeability was observed.

### 3.2. P-Glycoprotein Expression and Activity

#### *3.2.1. Immunoblotting*

The western blot analysis revealed a C219 antibody reactive band of 170kD in both the sonicated and non-sonicated cells, corresponding to P-gp which is markedly overexpressed in our Caco-2 cell line (Fig. (**3**)).

#### *3.2.2. Transepithelial Transport of P-gp Substrates*

P-glycoprotein activity was confirmed in our Caco-2 cell monolayers by measuring the bidirectional transport of several P-gp probe substrates and the effect of selective and non-selective P-gp inhibitors on their transport. Rhodamine (5µM) [28] and digoxine (5µM) [29] were used as well-known P-gp substrates. Cyclosporine A (10µM) [22] and GF120918 (4µM) [23] were used as potent first and second-generation P-gp inhibitors. Substrates interaction with P-gp was examined at pH conditions reflecting the gastrointestinal tract (pH: 6.8/7.4) and at physiological pH reflecting the pH surrounding the blood-brain-barrier (pH: 7.4/7.4).

*Rhodamine 123*: The transepithelial transport of rhodamine 123 was more than 2-fold higher from the basolateral to apical side (B-A) than from the apical to basolateral (A-B) at both pH conditions (pH 6.8/7.4 and 7.4/7.4). The addition of cyclosporine A, a potent P-gp inhibitor, decreased the B-A transport by 66 to 85% and increased the A-B transport by 1.8-fold average ($p<0.01$). The efflux ratios (TRs) were not significantly different at the two pH conditions tested. (Table **1**, Fig. (**4**)).

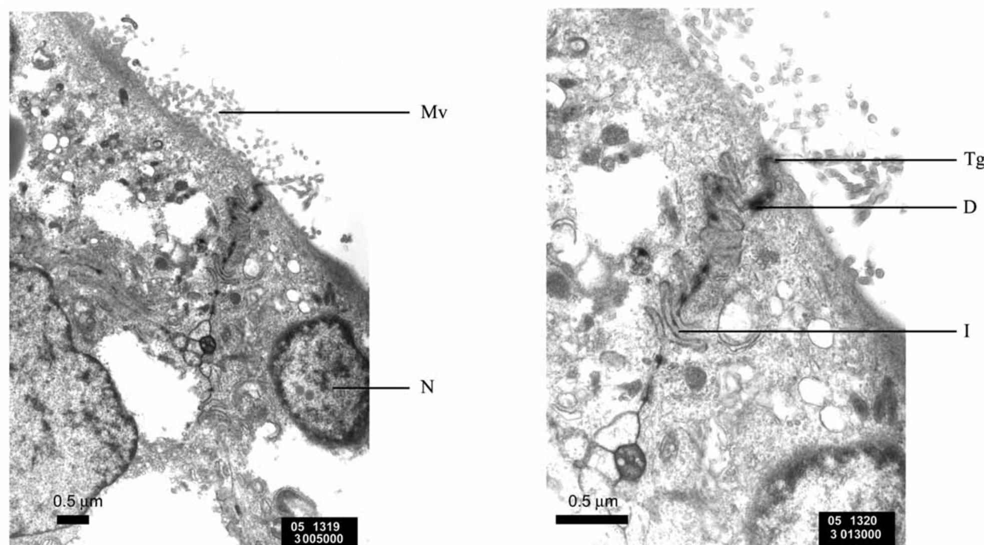


**Fig. (2).** Low and high power TEM micrograph of Caco-2 cell monolayers epithelium at day 21.
Mv: Microvilli, N:nucleus, I: interdigitation, D: desmosome, Tg: tight junction.

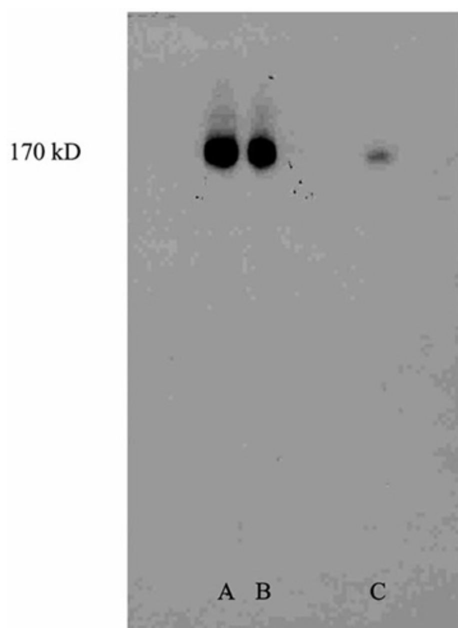


**Fig. (3).** Western Blot analysis of P-glycoprotein expression in Caco-2 cells monolayers at day 21.
(A) Non-sonicated Caco-2 cells. (B) Sonicated Caco-2 cells. (C) Non-sonicated supernatant.

*Digoxine:* At pH 6.8/7.4, we observed a significant efflux ratio with a B-A transport nearly 6-fold greater than the A-B one. The addition of GF120918, a specific and potent P-gp inhibitor, increased the absorptive influx by 2-fold and decreased the secretory efflux by 60%. At physiological pH, the efflux ratio was greater with a B-A transport 25-fold greater than the A-B one. A similar 60% inhibition of the B-A transport and more than a 4-fold increase of the A-B transport were observed in the presence of GF120918 ($p<0.01$) (Table **1**, Fig. (**4**)).

### 3.3. Transepithelial Transport of Dextromethorphan and Dextrorphan

The bidirectional transport of dextromethorphan and dextrorphan in the presence and absence of P-gp inhibitors, at two pH conditions, was investigated at respectively 0.25-150 and 1-500 times the plasma concentrations that might produce neuroprotective anti-NMDA effect *in vivo* (1 to 100μM) [30].

#### *3.3.1. Transepithelial Transport of Dextromethorphan*

At pH 6.8/7.4 conditions, a significant efflux ratio was observed with a B-A transepithelial transport 2 to 3-fold higher than the A-B one. At physiological pH, the differential transport was nearly abolished (TR:1-1.4). Neither the A-B nor the B-A transports were affected by cyclosporine (Table **1**, Fig. (**5**)), except a slight increase of both the A-B and B-A transport of 1μM DEM at physiological pH. A slight increase of the B-A and A-B transport of DEM 10 and 100μM respectively at a pH gradient was also observed ($p<0.01$). The transepithelial transport of dextromethorphan was not modulated by GF120918 at the lower concentration (1μM) under the two pH conditions ($p<0.01$) (Table **1**).

#### *3.3.2. Transepithelial Transport of Dextrorphan*

As for DEM, a significant efflux ratio was observed at pH 6.8/7.4 conditions, with a transepithelial transport 2 to 3-fold higher from the basolateral to apical side (B-A) than the apical to basolateral (A-B). At physiological pH, the differential transport was also nearly abolished (TR: 1-1.4) and neither the A-B nor the B-A transports were affected by cyclosporine in the two pH conditions (Table **1**, Fig. (**5**)), except a slight increase of the B-A transport of DOR 50μM at the pH gradient ($p<0.01$). Transepithelial transport of dextrorphan was not modulated by GF120918 at the lower concentration (1 μM) under the two pH conditions ($p<0.01$) (Table **1**).

## 4. DISCUSSION

Our results strongly suggest that DEM and DOR are not P-gp substrates. DEM and DOR showed in fact a significant permeability ratio (TR: 2-3; $p<0.01$) in the presence of a pH gradient, suggesting an efflux transport of the two drugs. However, at physiological pH, the polarized transport of the two drugs was nearly abolished (TR: 1-1.4; $p<0.01$). Rhodamine and digoxine, two well-known P-gp substrates, confirmed P-gp-mediated efflux activity in our Caco-2 cell model. Typically, the polarized transepithelial transport observed across the Caco-2 cell monolayers at the two pH conditions as well as the increased absorptive and decreased secretory flux of the two probes in the presence of a P-gp inhibitor demonstrates the presence of a functional P-gp (Table **1**, Fig. (**4**)). The lack of effect of the P-gp inhibitor cyclosporine as well as GF120918 on both the absorptive and secretory transports pathways of DEM and DOR at both pH conditions (Table **1**, Fig. (**5**)) attests that the two NMDA antagonists are not P-gp substrates at respectively 0.25-150 and 1-500 times the plasma concentrations that might produce neuroprotective anti-NMDA effect *in vivo* [30].

Our results showing that DEM is not a P-gp substrate is in concordance with its pharmacokinetic profile. In fact, DEM is almost completely absorbed by the gastrointestinal tract and its brain-to-plasma ratio ranges from 25 to 500 [1]. Steinberg *et al.* [3] showed a good intestinal absorption and brain accumulation of DEM in a clinical trial involving neurosurgical patients receiving high oral doses of dextromethorphan. DEM brain levels were more than 13-fold higher than those of DOR and DOR brain-to-plasma ratio 5-fold lower than that of DEM suggesting a poor brain bioavailability of the metabolite. DEM and DOR protein binding factor could not be taken in account as data are still lacking. Our data shows a higher DEM absorptive apparent permeability coefficient ($P_{app}$(A-B)) than DOR over the concentration range tested. As our study shows that DEM and DOR are not P-gp substrates, the differential brain penetration and thus pharmacodynamic effect of the two drugs could be related to their different physico-chemical properties. Indeed, DEM has a higher liposolubility (LogP: 4.11 ± 0.4) as compared to DOR (LogP: 3.46 ± 0.3) and this may contribute to a lower cerebral bioavailability and anti-NMDA neuromodulatory effect of DOR as compared to DEM.

The gastrointestinal tract contains stomach acid and additional pH-mediated efflux mechanisms such as the intestinal $Na^+$-$H^+$ antiporters, both of which are responsible for its acidic micro-climate and pH gradient properties [31]. Caco-2 cells have been shown to exhibit active $Na^+$-$H^+$ antiporters, located on the basolateral side of differentiated cells [32]. Those transporters, among other $H^+$ antiport systems, have been shown to be involved in the gastrointestinal secretory efflux of drugs such as loperamide, diphenydramine and zolmitriptan [33-35]. Indeed, the pH gradient has been suggested to play a significant role in preventing the systemic absorption of the antidiarrheal agent loperamide in parallel to P-gp [34]. DEM and DOR's efflux ratio observed at gastrointestinal pH (Table **1**) is probably linked to pH-mediated efflux mechanisms such as the above-mentioned $Na^+$-$H^+$ exchangers. Indeed, the degree of ionisation of DEM and DOR is pH-dependant (pKa: 9.13 ± 0.2 and 8.89 ± 0.2, respectively). Hence, at pH 6.8, the two molecules are entirely ionized and may interact with the $Na^+$-$H^+$ exchangers. P-gp is not affected by extracellular pH [36] and this data clearly implies that the efflux observed is not attributed to any P-gp pH-related modification. On the other hand, rhodamine has a lower pKa (pKa: 6.12 ± 0.4) than DEM and DOR and at pH 6.8, the low percentage of the ionized form (~20%) makes P-gp its predominant efflux transporter. That explains the similar transport ratio observed at both pH conditions. Digoxine is entirely non-ionized at both pH conditions (pKa: 13.5 ± 0.7). Thus, P-gp and possibly another ef-

**Table 1. Apparent Permeability Coefficients Papp (cm/s) of Rhodamine, Digoxine, Dextromethorphan and Dextrorphan in the Presence and Absence of P-gP Inhibitors at pH 6.8/7 and pH 7.4/7.4**

| Drug Concentration ± P-gp inhibitors | $P_{app}$ (A-B) $P_{app}$ (B-A) (cm/s) ($10^{-6}$) pH 6.8/7.4 | Efflux ratio (TR) | $P_{app}$ (A-B) $P_{app}$ (B-A) (cm/s) ($10^{-6}$) pH 7.4/7.4 | Efflux ratio (TR) |
|---|---|---|---|---|
| **Rhodamine** | | | | |
| 5µM | 3.7 ± 1.9 7.9 ± 1.4[a] | 2.1 (efflux) | 2.1 ± 0.1 5.5 ± 0.8[a] | 2.6 (efflux) |
| + 10µM Cyclosporine | 7.1 ± 1.4[b] 1.1 ± 0.2[b] | 0.1 | 3.9 ± 0.9[b] 1.8 ± 0.4[b] | 0.4 |
| **Digoxine** | | | | |
| 5µM | 1.9 ± 0.6 10.8 ± 0.9[a] | 5.6 (efflux) | 1.1 ± 0.1 27.7 ± 2.8[a] | 25.1 (efflux)[c] |
| + 4µM GF120918 | 3.3 ± 0.3[b] 4.3 ± 0.2[b] | 1.3 | 4.7 ± 0.7[b] 10.1 ± 0.3[b] | 2.1 |
| **Dextromethorphan** | | | | |
| 1 µM | 21.6 ± 1.3 62.6 ± 2.1[a] | 2.8 (efflux) | 12.1 ± 0.4 16.5 ± 0.3[a] | 1.3 (no net flux)[c] |
| + 10 µM Cyclosporine | 22.5 ± 1.8 63.4 ± 3.0 | 2.8 | 15.4 ± 0.2[b] 19.2 ± 0.4[b] | 1.2 |
| 10 µM | 20.3 ± 0.3 52.8 ± 0.5[a] | 2.6 (efflux) | 32.1 ± 0.6 33.1 ± 1.1 | 1.0 (no net flux)[c] |
| + 10 µM Cyclosporine | 21.3 ± 0.3 56.6 ± 0.9[b] | 2.6 | 33.3 ± 1.2 37.3 ± 1.9 | 1.1 |
| 50 µM | 15.5 ± 0.3 39.1 ± 2.0[a] | 2.5 (efflux) | 27.0 ± 1.3 37.2 ± 0.7[a] | 1.3 (no net flux)[c] |
| + 10 µM Cyclosporine | 14.9 ± 0.3 40.5 ± 0.3 | 2.7 | 27.9 ± 4.9 39.5 ± 1.0 | 1.4 |
| 100 µM | 16.7 ± 0.3 53.2 ± 3.7[a] | 3.1 (efflux) | 28.1 ± 0.2 39.6 ± 0.5[a] | 1.4 (no net flux)[c] |
| + 10 µM Cyclosporine | 18.1 ± 0.2[b] 48.6 ± 4.0 | 2.6 | 26.8 ± 0.6 41.6 ± 0.7 | 1.5 |
| 1 µM | 15.3 ± 0.4 31.3 ± 1.0[a] | 2.0 (efflux) | 20.7 ± 1.6 22.7 ± 0.6 | 1.0 (no net flux)[c] |
| + 4 µM GR120918 | 16.7 ± 0.9 31.9 ± 2.1 | 1.9 | 20.1 ± 1.3 23.1 ± 0.8 | 1.1 |
| **Dextrorphan** | | | | |
| 1 µM | 18.7 ± 1.3 38.0 ± 3.2[a] | 2.0 (efflux) | 19.9 ± 1.1 21.8 ± 1.0 | 1.0 (no net flux)[c] |
| + 10 µM Cyclosporine | 17.9 ± 1.7 38.6 ± 1.1 | 2.1 | 19.7 ± 0.6 22.5 ± 0.2 | 1.1 |
| 10 µM | 15.5 ± 0.8 42.4 ± 2.0[a] | 2.7 (efflux) | 14.3 ± 0.7 18.3 ± 1.2[a] | 1.2 (no net flux)[c] |
| + 10 µM Cyclosporine | 16.8 ± 0.6 34.9 ± 2.7 | 2.0 | 14.7 ± 1.1 17.7 ± 0.7 | 1.2 |
| 50 µM | 17.5 ± 1.8 37.1 ± 1.2[a] | 2.1 (efflux) | 14.9 ± 0.3 20.4 ± 0.8[a] | 1.3 (no net flux)[c] |
| + 10 µM Cyclosporine | 14.8 ± 2.0 41.3 ± 0.8[b] | 2.7 | 15.3 ± 0.7 19.8 ± 0.4 | 1.2 |
| 100 µM | 16.6 ± 0.4 47.3 ± 1.3[a] | 2.8 (efflux) | 26.4 ± 0.9 37.2 ± 1.0[a] | 1.4 (no net flux)[c] |
| + 10 µM Cyclosporine | 17.0 ± 0.6 51.1 ± 2.0 | 3.0 | 26.8 ± 0.9 37.5 ± 2.0 | 1.3 |
| 1 µM | 14.5 ± 1.6 30.7 ± 1.0[a] | 2.1 (efflux) | 20.2 ± 1.6 25.7 ± 0.4[a] | 1.2 (no net flux)[c] |
| + 4 µM GR120918 | 15.1 ± 0.9 29.2 ± 2.0 | 1.9 | 20.7 ± 1.4 24.5 ± 1.2 | 1.1 |

[a]p<0.01. Transport difference in each direction.
[b]p<0.01. Inhibitor addition in relation to controls for a particular direction.
[c]p<0.01. Transport efflux ratio difference in relation to pH conditions.
Values are the mean ± SD of 3 experiments.

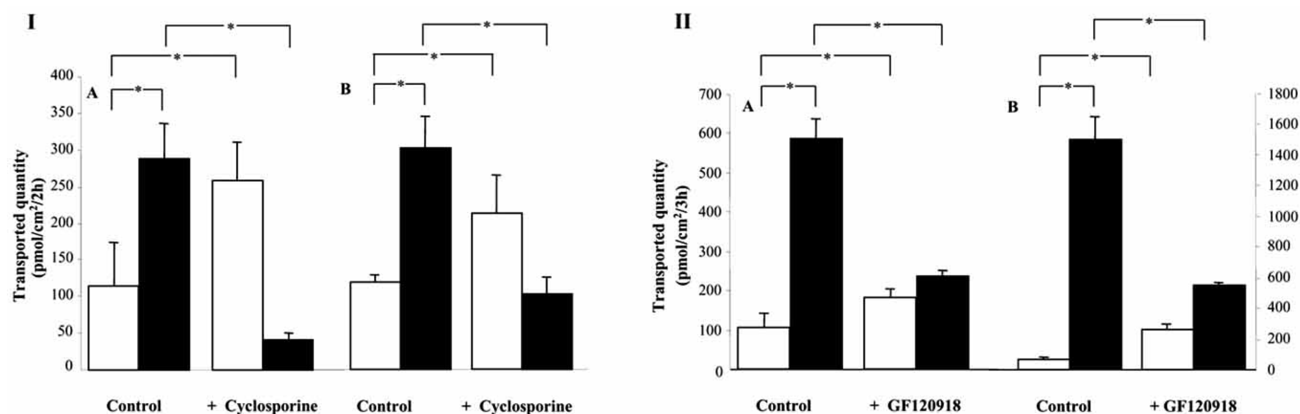


**Fig. (4).** Transepithelial transport of rhodamine (I) and digoxine (II) across Caco-2 cell monolayers, the effect of (P-gP inhibitors) A and the influence of pH conditions.
Rhodamine (I) or digoxine (II) (5µM) were added to the apical side (A) (open columns) or the basolateral side (B) (solid columns) of the monolayers in the presence (cyclosporine or GF120918) or absence (control) of the inhibitors at pH 6.8/7.4 (Graph A) and pH 7.4/7.4 (Graph B). Data are the mean ± SD of 3 experiments. *p<0.01.

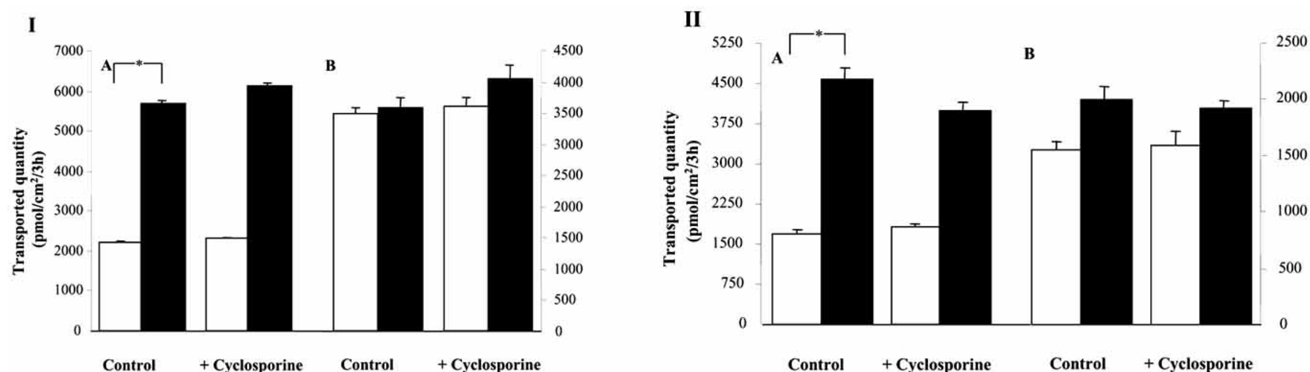


**Fig. (5).** Transepithelial transport of dextromethorphan (I) and dextrorphan (II) across Caco-2 cell monolayers, the effect of cyclosporine A and the influence of pH conditions.

Dextromethorphan (I) and dextrorphan (II) (10µM) were added to the apical side (A) (open columns) or the basolateral side (B) (solid columns) of the monolayers in the presence (+ cyclosporine) or absence (control) of 10µM cyclosporine A at pH 6.8/7.4 (Graph A) and pH 7.4/7.4 (Graph B). Data are the mean ± SD of 3 experiments. *p<0.01.

flux transporter [37] are expected to be the only contributors to its secretory efflux under pH-gradient conditions.

Caco-2 cell monolayers are a widely used *in vitro* model in drug development and have lead to optimisation as a predictive tool for drug transport by P-gp. Caco-2 cells are human colorectal adenocarcinoma cells surexpressing P-gp. They spontaneously differentiate when grown on porous membranes to form monolayers of polarized cells that function similarly to intestinal enterocytes [38]. This model is in fact often used as an *in vitro* predictive tool for drug intestinal absorption [38-40] and the predictive value of the Caco-2 cell model with respect to blood-brain barrier (BBB) distribution has been criticized, principally in the context of studies comparing it with animal *in vivo* studies [41,42]. However, several studies showed a good correlation between drug permeability ratio across Caco-2 cell monolayers and brain penetration in rodents [43,44]. Moreover, it is important to specify that the aim of our study was not to predict the overall BBB penetration of DEM and DOR but to evaluate as closely as possible the specific interaction of these two molecules with the human P-glycoprotein. In this context, the Caco-2 cell model, as well as many other animal *in vitro* cell models, including the ABCB1-transfected Madin-Darby canine kidney (MDCK) and porcine kidney-derived cell line (LLC-PK1) have been widely used to estimate drug affinity, notably opioids, with P-gp and extrapolate it with their blood-brain-barrier penetration [45-47]. Moreover, most established *in vitro* models of blood-brain barrier use cells of non human origin, which is not optimal for the prediction of brain permeability in human. Actually, except the available drug transport study across the co-culture-based model of human BBB developed by Megard and al. [48], which has not been reproduced ever since, *in vitro* studies focus generally only on drug accumulation into human brain endothelial cells [49].

Furthermore, a recent study confirmed that dextromethorphan is not a P-gp substrate and suggested that other efflux transporters may be implicated [50]. However, indirect evidences support that dextromethorphan could be a P-gp substrate. A clinical trial showed an increased bioavaibility of DEM when co-administrated with grapefruit and Seville orange juices, two P-gp inhibitors that are also CYP3A4 inhibitors [51-53]. CYP 3A4 expression in Caco-2 cells is still controversial [54,55], however, as cyclosporine is both a potent CYP3A4 and P-gp inhibitor and because of the overlap in substrate specificity between CYP3A4 and P-gp [56], the eventual formation of drug metabolites under normal conditions in our Caco-2 cells was checked in order to avoid any overlap inhibition. No methoxymorphinan or other metabolite was detected by HPLC, confirming the absence of significant CYP3A4 activity in our Caco-2 cell model. A role of P-gp in the cerebral uptake of DEM has also been suggested by an animal study showing a 2-fold increase of DEM concentration in the brain of abcb1ab (-/-) knockout mice as compared to wild-type mice [57]. However, inter-species differences are well-known and a difference in P-gp substrate recognition and transport efficacy between human and rodents, at least for certain compounds, has been well demonstrated [58,59]. Another study showed an enhanced cerebral uptake of dextromethorphan in rats by concomitant administration of verapamil but no effect on its systemic biodisposition [60]. According to these results, it was suggested that dextromethorphan might possibly interact with transporters other than P-gp, possibly present at the BBB but not at the intestinal barrier. The BBB and the intestinal tract share nearly the same influx and efflux transporters [61]. Nevertheless, a novel transporter, the brain multidrug resistance protein (BMDP) has been recently characterized in porcine brain capillary endothelial cells (PBCEC). BMDP was shown to be highly homologous to the human breast cancer resistance protein (BCRP/ABCG2) and more strongly expressed in the capillary endothelial cells than P-gp and MRP1 [62,63]. This transporter or another animal cerebral transporter could be responsible for the observed DEM transport. The latter study showed no influence of P-gp or any other efflux transporter on plasmatic or cerebral DOR pharmacokinetics, therefore confirming our results.

Except for the last hypothesis that DEM may be substrate of a transporter present at the BBB but not at the intestinal barrier, other hypothesis may be advanced. Caco-2 cells express a large variety of efflux drug transporters as showed by Taipalensuu *et al.* [39], transporters which are almost all inhibited by cyclosporine with the exception of the multi-resistance proteins MRP3 to MRP6 [16,61]. Those transporters are also not inhibited by GF120918 [61]. Otherwise, the two NMDA antagonists may interact with the efflux drug transporter BCRP which is expressed in an extremely low extent in Caco-2 cells comparing to the intestinal and blood-brain barrier [33,61,64]. Further studies are needed to explore the role of these efflux transporters in the transepithelial transport of dextromethorphan and its active metabolite.

In conclusion, we present the first *in vitro* study of the interaction of dextromethorphan and dextrorphan with the human P-glycoprotein. Our data indicate that neither dextromethorphan nor dextrorphan are P-gp substrates but pH-mediated efflux mechanisms, probably $Na^+$-$H^+$ exchangers, may be involved in limiting dextromethorphan gastrointestinal absorption and dextrorphan transmembranal passage when a pH gradient is present. The preferential anti-N-Methyl-D-Aspartate neuromodulatory effect of dex-

tromethorphan, which accumulates in the central nervous system *in vivo*, seems not to be related to a central P-gp efflux of DOR but possibly to a higher liposolubility of DEM.

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## ABBREVIATIONS

NMDA = N-methyl-D-aspartate
DEM = Dextromethorphan
DOR = Dextrorphan
HBSS = Hank's balanced salt solution
HEPES = N-(2-hydroxylethyl)piperazine-N'-2ethane-sulfonic acid
MES = 32-(N-morpholino)ethane-sulfonic acid
GF120918, Elacridar = [N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide]
A-B = Apical-to-basolateral
B-A = Basolateral-to-apical
Papp = Apparent permeability coefficient
HPLC = High-performance liquid chromatography.

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