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Role of blood-brain barrier P-glycoprotein in limiting brain accumulation and sedative side-effects of asimadoline, a peripherally acting analgaesic drug

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1 Studies with knockout mice lacking mdr1a P-glycoprotein (P-gp) have previously shown that blood-brain barrier P-gp is important in preventing the accumulation of several drugs in the brain.

2 Asimadoline (EMD 61753) is a peripherally selective κ-opioid receptor agonist which is under development as a therapeutic analgaesic. From the structural characteristics of this drug and its peripheral selectivity, we hypothesized that it is transported by P-gp.

3 Using a pig-kidney polarized epithelial cell line transfected with *mdr* cDNAs, we demonstrate that asimadoline is transported by the mouse mdr1a P-gp and the human MDR1 P-gp.

4 Furthermore, we show that in *mdr1a/1b* double knockout mice, the absence of P-gp leads to a 9 fold increased accumulation of asimadoline in the brain. In line with this accumulation difference, *mdr1a/1b* (−/−) mice are at least 8 fold more sensitive to the sedative effect of asimadoline than wild-type mice.

5 Interestingly, the oral uptake of asimadoline was not substantially altered in *mdr1a/1b* (−/−) mice.

6 Our results demonstrate that for some drugs, P-gp in the blood-brain barrier can have a therapeutically beneficial effect by limiting brain penetration, whereas at the same time intestinal P-gp is not a significant impediment to oral uptake of the drug.

Keywords: P-glycoprotein; asimadoline (EMD 61753); κ-opioid receptor agonist; blood-brain barrier; drug disposition; oral availability

Abbreviations: CID, collision induced dissociation; CNS, central nervous system; P-gp, P-glycoprotein; SRM, selected reaction monitoring; TFA, trifluoroacetic acid

Introduction

A potential problem in the use of peripherally acting therapeutic drugs is, in the case of opioids, the occurrence of an unwanted central nervous system (CNS) side-effects including sedation, respiratory depression, dependence, itch, nausea and dysphoria (reviewed in Stein & Schäfer, 1997). Therefore, new generations of drugs are currently being developed with limited ability to penetrate the blood-brain barrier. One of these drugs is the peripherally-selective κ-opioid receptor agonist asimadoline (EMD 61753). Asimadoline is peripherally effective against hyperalgaesic pressure and neurogenic inflammatory pain stimuli, probably by interfering with peripheral opioid receptors. Asimadoline has only a limited ability to penetrate the blood-brain barrier, and thus a limited capacity to elicit CNS-mediated sedation, aversion, diuresis and analgaesia (Barber *et al*., 1994; Gottschlich *et al*., 1995). It is known that the drug-transporting P-glycoprotein (P-gp) in the blood-brain barrier limits the entry of various drugs into the brain (Schinkel *et al*., 1994; 1995; 1996). The hydrophobic, amphiphilic character of asimadoline and its peripheral pharmacological selectivity led us to hypothesize that this drug is a P-gp substrate.

P-gps were originally discovered in cancer cells where they confer multidrug resistance (Juliano & Ling, 1976). They are 140–170-kDa plasma membrane proteins that actively extrude a wide range of amphiphilic and hydrophobic drugs from the cell (Juliano & Ling, 1976; Gros *et al*., 1986; Chen *et al*., 1986; Endicott & Ling, 1989; Gottesman & Pastan, 1993). Humans have one gene, *MDR1*, encoding a drug-transporting P-gp, while mice have two genes, *mdr1a* (also called *mdr3*) and *mdr1b* (also called *mdr1*). The tissue distribution of the mouse mdr1a and mdr1b P-gp suggests that together they fulfil the same function as the MDR1 P-gp in humans (reviewed by Borst & Schinkel, 1997). Previous studies with knockout mice lacking mdr1a P-gp and other animal models, have shown that blood-brain barrier P-gp is important in preventing the brain from accumulating a range of drugs (Schinkel *et al*., 1994; 1995; 1996; Drion *et al*., 1996; Huwyler *et al*., 1996; Mayer *et al*., 1996; Kim *et al*., 1998). In mice, mdr1a P-gp is the most prominent, if not the only, P-gp isoform present at the blood-brain barrier and in the intestinal epithelium (Schinkel *et al*., 1994; 1997). In addition, mdr1a P-gp in the intestine diminishes the oral bioavailability and increases the direct intestinal excretion of several drugs (Fricker *et al*., 1996; Mayer *et al*., 1996; Sparreboom *et al*., 1997; Kim *et al*., 1998),

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and both mdr1a and (to a lesser extent) mdr1b P-gp can contribute to the hepatobiliary excretion of a range of drugs (Smit *et al*., 1998a).

In this study we show that the peripherally selective κ-opioid receptor agonist asimadoline is transported by the mouse mdr1a and human MDR1 P-gp. We further made use of the *mdr1a/1b* knockout mouse model (Schinkel *et al*., 1997) to determine the effect of absence of the mrd1a and mdr1b P-gps on the sedative effect, tissue distribution and excretion of this drug *in vivo*.

Methods

Animals

The animals that were used in all experiments were male wild-type and *mdr1a/1b* (−/−) mice of a 99% FVB genetic background, between 9–14 weeks of age. Animals were kept under controlled temperature with a 12 h/12 h light/dark cycle. They received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*.

Chemicals

[^{14}C]-asimadoline (5.69 mCi mmol^{-1}) and unlabelled asimadoline were provided by Merck (Grafing, Germany). [^{3}H]-inulin (0.8 Ci mmol^{-1}) was obtained from Amersham Life Science (Little Chalfont, U.K.). Hypnorm was from Janssen Pharmaceuticals B.V. (Tilburg, The Netherlands). Dormicum was from Roche Nederland B.V. (Mijdrecht, The Netherlands). Metofane (methoxyflurane) was from Mallinckrodt Veterinary, Inc. (Mundelein, IL, U.S.A.). L-glutamine was from GIBCO BRL (Paisley, Scotland, U.K.). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cells and tissue culture

The pig-kidney epithelial cell line LLC-PK1 was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The generation of *MDR1*-, *mdr1a*- and *mdr1b*-transfected LLC-PK1 subclones L-*MDR1*, L-*mdr1a* and L-*mdr1b* was described previously (Schinkel *et al*., 1995; Smit *et al*., 1998b). Cells were cultured in M199 medium supplemented with L-glutamine, penicillin (50 U ml^{-1}), streptomycin (50 μg ml^{-1}) and 10% (v v^{-1}) foetal calf serum ('complete serum') at 37°C in the presence of 5% CO_2. Cells were subcultured by trypsinization every 3–4 days.

Transport assays

Transport assays were carried out as described earlier (Schinkel *et al*., 1995) with minor modifications. Complete medium including L-glutamine, penicillin, streptomycin, and foetal calf serum was used throughout. Cells were seeded on microporous polycarbonate membrane filters (3.0 μm pore size, 24.5-mm diameter, Transwell® 3414, Costar®) at a density of 2×10^6 cells per well. The cells were grown for 3 days in complete medium with one medium replacement. 1–2 h before the start of the experiment, medium at both the apical and the basal side of the monolayer was replaced with 2 ml of complete medium. The experiment was started ($t = 0$) by replacing the medium at either the apical or the basal side of the cell layer with 2 ml of complete medium containing 10 μM of [^{14}C]-asimadoline (3.23 Ci mol^{-1}), and 192 nM of [^{3}H]-inulin (0.8 Ci mmol^{-1}). The cells were incubated at 37°C in 5% CO_2, and 50 μl aliquots were taken every hour from each compartment. The appearance of radioactivity in the opposite compartment was measured and presented as the fraction of total radioactivity added at the beginning of the experiment. Under these experimental conditions, 1% translocation of asimadoline per hour corresponds to a permeability coefficient of 0.042 mm h^{-1}. The tightness of the monolayer was measured by monitoring the paracellular flux of [^{3}H]-inulin to the opposite compartment. This flux was always lower than 1.5% of the total radioactivity per hour.

Pharmacokinetics

For intravenous administration of asimadoline, 5 μl drug solution (appropriate concentration in 5% (w v^{-1}) D-glucose) per g body weight was injected into the tail vein of mice lightly anaesthetized with diethyl ether. For oral administration, 10 μl drug solution (appropriate concentration in 5% D-glucose) per g body weight was dosed by gavage into the stomach. Animals were sacrificed by cardiac puncture or axillary bleeding after anaesthesia with Metofane (methoxyflurane). Organs and tissues were removed and homogenized in 4% (w v^{-1}) bovine serum albumin (BSA). Levels of radioactivity in homogenates were determined as described (Mayer *et al*., 1996).

Sedation experiments

Sedation times were determined after intravenous injection of asimadoline into the tail vein of mice lightly anaesthetized with diethyl ether. Mice generally recovered from diethyl ether anaesthesia alone within 10–20 s. The sedation time due to asimadoline was defined as the period after administration of asimadoline during which the animal remained passive and showed no spontaneous explorative behaviour.

Excretion experiments

Mice were anaesthetized and cannulated as described (Mayer *et al*., 1996) with minor adjustments.

Anaesthesia a combination of Hypnorm (fentanyl 0.2 mg ml^{-1}, fluanisone 10 mg ml^{-1}) and Dormicum (midazolam 5 mg ml^{-1}) was used as a mixture of two parts 0.3 M glucose, one part Hypnorm and one part Dormicum. The volume of the anaesthetic solution injected intraperitoneally was 7 μl g^{-1} body weight.

Gall-bladder cannulation after opening of the abdominal cavity and distal ligation of the common bile duct, a polythene catheter (Portex Limited, Hythe, U.K.), with an inner diameter of 0.28 mm, was inserted into the incised gall-bladder. The catheter was fixed to the gall-bladder with an additional ligation. Bile was collected for 60 min after intravenous injection of [^{14}C]-asimadoline into the tail vein. At the end of the experiment, blood was collected by axillary bleeding. During the experiment the urinary bladder was allowed to fill with urine. Urine was collected by ligation of the urinary bladder just before sacrificing the animal. Subsequently, the complete bladder was removed and emptied. Organs and tissues were removed and homogenized in 4% (w v^{-1}) BSA. Levels of radioactivity in homogenates were determined as described (Mayer *et al*., 1996).

Determination of unchanged asimadoline

Unchanged asimadoline (EMD 61753) was determined using D_4-EMD 61753 as a stable isotope internal standard in plasma, brain tissue and bile by a sensitive and selective LC-MS/MS quantitative assay.

Sample work-up procedures Plasma: to a sample of 50 μl plasma, 50 μl of an internal standard solution (200 pg μl^{-1} D_4-EMD 61753, 1 M ammoniumacetate buffer pH 7) and 750 μl of diisopropylether were added in a suitable test tube. The tubes were shaken for 10 min and centrifuged at approximately 5000 r.p.m. The aqueous layer was frozen and the organic layer was transferred into a clean test tube. The organic layer was evaporated to dryness and the residue was dissolved in 1 ml methanol/0.01% trifluoroacetic acid (TFA) = 10/90 (v v^{-1}). An aliquot of 20 μl was injected onto the HPLC column. Brain: To a sample of 50 μl brain extract, 50 μl of an internal standard solution (200 pg μl^{-1} D_4-EMD 61753, 1 M ammoniumacetate buffer pH 7) and 750 μl of tert-butyl methyl ether (MTBE) were added in a suitable test tube. The tubes were shaken for 10 min and centrifuged at approximately 5000 r.p.m. The aqueous layer was frozen and the organic layer was transferred into a clean test tube. The organic layer was evaporated to dryness and the residue was dissolved in 1 ml methanol/0.01% TFA = 10/90 (v v^{-1}). An aliquot of 20 μl was injected onto the HPLC column. Bile: Bile samples were diluted 100 fold with methanol/0.01% TFA = 10/90 (v v^{-1}). To an aliquot of 200 μl diluted bile sample 50 μl of an internal standard solution (20 pg μl^{-1} D_4-EMD 61753, 1 M ammoniumcetate buffer pH 7) was added. An aliquot of 50 μl was injected onto the HPLC column.

For liquid chromatographic analysis the system consisted of a Merck Hitachi Pump L-7100 and a Merck Hitachi Autosampler L-7250. As analytical HPLC column Superspher 100 RP-18e, 4 μm, 20 × 2 mm I.D. from Merck KGaA, Darmstadt, Germany was employed. As mobile phase methanol/0.02% TFA = 60/40 (v v^{-1}) at isocratic conditions with a flow rate of 500 $\mu l\ min^{-1}$ was used at ambient temperature. Under these conditions the retention time of both EMD 61753 and D_4-EMD 61753 was approximately 0.35 min.

For mass-spectrometric detection a Perkin-Elmer SCIEX (Thornhill, Ontario, Canada) model API 3000 triple quadrupole mass spectrometer equipped with a Turbo-Ion interface was used. For SRM-LC-MS, the analytical column was coupled directly to the Turbo-Ion interface, which was maintained at a temperature of 450°C. The ion spray and orifice voltages were set at 4500 and 35 V, and positive ions were sampled into the quadrupole mass analyser. Ultrapure nitrogen was used as the curtain and nebulizing gas flow rates of 1.25 and 1.04 l min^{-1}. Prior to each analytical sequence the first separation quadrupole (Q1) was tuned using a standard solution of EMD 61753 and D_4-EMD 61753 to yield a resolution of 0.7 Da at half peak height for the precursor ion mass peaks of the protonated molecules at m/z 415.1 and 419.1, respectively. The same procedure was repeated with the second separation quadrupole (Q3) using the product ion scan mode to yield a resolution of 0.7 Da at half peak height for the corresponding product ions at m/z 328.0 and 332.0.

EMD 61753 and its tetradeuterium-labelled internal standard were detected in the positive ion mode by selected reaction monitoring (SRM). The mass transitions of m/z 415.1 to 328.0 and m/z 419.1 to 332.0, respectively were used for SRM. The dwell time was 200 ms for both analytes, associated with a pause time of 2 ms. For CID (collision-induced dissociation), nitrogen was used at a thickness of about 260×10^{13} atoms cm^{-2}, the collision energy for fragmentation of the precursor ions was set at 24 eV.

The limit of quantitation for EMD 61753 in plasma was 5 ng ml^{-1}. Within the concentration range of 8–4000 ng ml^{-1}, the deviation of the quality control samples from the target concentration was 6.01% or less. According to the analysis of the calibration samples the assay was linear with a correlation coefficient (r) of 0.9997. The limit of quantitation for EMD 61753 in brain tissue was 1 ng ml^{-1} brain tissue extract. Within the concentration range of 8–4000 ng ml^{-1}, the deviation of the quality control samples from the target concentration was 4.54% or less. According to the analysis of the calibration samples the assay was linear with a correlation coefficient (r) of 0.9996. The limit of quantitation of EMD 61753 in bile was 5 ng ml^{-1}. Within the concentration range of 16–400 ng ml^{-1}, the deviation of the quality control samples from the target concentration was 8.51% or less. According to the analysis of the calibration samples the assay was linear with a correlation coefficient (r) of 0.9983.

Statistical analysis

All values are given as means ± s.d. A two-tailed Student's t-test was used to assess the significance of difference between two sets of data. Differences were considered to be statistically significant when $P < 0.05$.

Results

In vitro *transport of [^{14}C]-asimadoline by mouse* mdr1a *and* mdr1b*, and human* MDR1 *P-glycoprotein*

To test whether asimadoline can be transported by P-gp *in vitro*, we made use of the polarized pig-kidney epithelial cell line LLC-PK1, transfected with mouse *mdr1a* or *mdr1b* or human *MDR1* cDNA (Schinkel *et al*., 1995; Smit *et al*., 1998b). These transfected cell lines contain roughly similar levels of mdr1a, mdr1b and MDR1 P-gp. The cell lines (LLC-PK1, L-*mdr1a*, L-*mdr1b* and L-*MDR1*) were grown to confluent polarized monolayers on porous membrane filters, and polarized trans-epithelial transport of [^{14}C]-asimadoline (10 μM) was determined.

In the parental cell line (LLC-PK1), translocation of [^{14}C]-asimadoline from the basal to the apical compartment (b-a) and *vice-versa* (a-b) was similar and amounted to about 16% per h during the initial phase of the experiment, corresponding to a permeability coefficient of about 0.68 mm h^{-1} (Figure 1). L-*mdr1a* and L-*MDR1* cells showed a markedly increased translocation from basal to apical, and a decreased translocation from apical to basal. In L-*mdr1b* cells asimadoline translocation from basal to apical was only slightly higher than in the parental cell line. These results show that asimadoline is well transported *in vitro* by mouse mdr1a P-gp and also by human MDR1 P-gp but not efficiently by mouse mdr1b P-gp.

Tissue distribution of [^{14}C]-asimadoline in mdr1a/1b *(−/−) and (+/+) mice*

It has been shown previously that the absence of P-gp in mice can result in an altered tissue distribution and excretion of several drugs (Schinkel *et al*., 1994; 1995; 1996; 1997; Mayer *et al*., 1996; Sparreboom *et al*., 1997). We analysed the *in vivo* distribution of radioactivity in *mdr1a/1b* (+/+) and (−/−) mice 60 min after i.v. administration of 1 mg kg^{-1} [^{14}C]-

asimadoline (Table 1). The most pronounced difference between the *mdr1a/1b* (+/+) and (−/−) mice was seen in the brain: over a period of 1 h, the *mdr1a/1b* (−/−) mice accumulated 9 fold more radioactivity in the brain than the *mdr1a/1b* (+/+) mice. Also, a more than 3 fold higher accumulation of radioactivity was observed in the testis of *mdr1a/1b* (−/−) mice. At the same time, plasma levels of radioactivity and the levels in the other tissues measured were comparable between *mdr1a/1b* (+/+) and (−/−) mice. We also carried out a [^{14}C]-asimadoline tissue distribution experiment in *mdr1a* (−/−) mice, which only lack the mdr1a P-gp. As one might expect from the inefficient transport of asimadoline by the mdr1b P-gp *in vitro* (see Figure 1), the results obtained 1 h after intravenous asimadoline (1 mg kg^{-1}) administration were virtually indistinguishable from those obtained with *mdr1a/1b* (−/−) mice (data not shown).

CNS side-effects of asimadoline in mdr1a/1b *(−/−) and (+/+) mice*

One of the CNS side-effects of κ-opioid receptor agonists is sedation (reviewed in Stein & Schäfer, 1997). To determine the effect of absence of P-gp from the blood-brain barrier on sedative effects of asimadoline, we measured the sedation time after various intravenous dosages of asimadoline in *mdr1a/1b*

Table 1 Tissue levels of radioactivity in *mdr1a/1b* (+/+) and (−/−) mice at 60 min after intravenous injection of 1 mg kg^{-} [^{14}C]-asimadoline

Tissue	mdr1a/1b *(+/+)* (ng-eq g^{-1})	mdr1a/1b *(−/−)* (ng-eq g^{-1})	*ratio (−/−):(+/+)*
Brain	65±7	586±48	9.1**
Muscle	210±45	179±46	0.9
Heart	353±49	377±64	1.1
Kidney	1258±265	964±225	0.8
Liver	2902±731	3311±2722	1.1
Lung	1892±201	1669±313	0.9
Spleen	720±74	694±112	1.0
Testis	161±3	528±63	3.3**
Plasma	211±33	199±34	0.9

Results are expressed as mean [^{14}C]-concentrations (ng-equivalent g^{-1})±s.d., $n=4$; $^{**}P<0.01$.

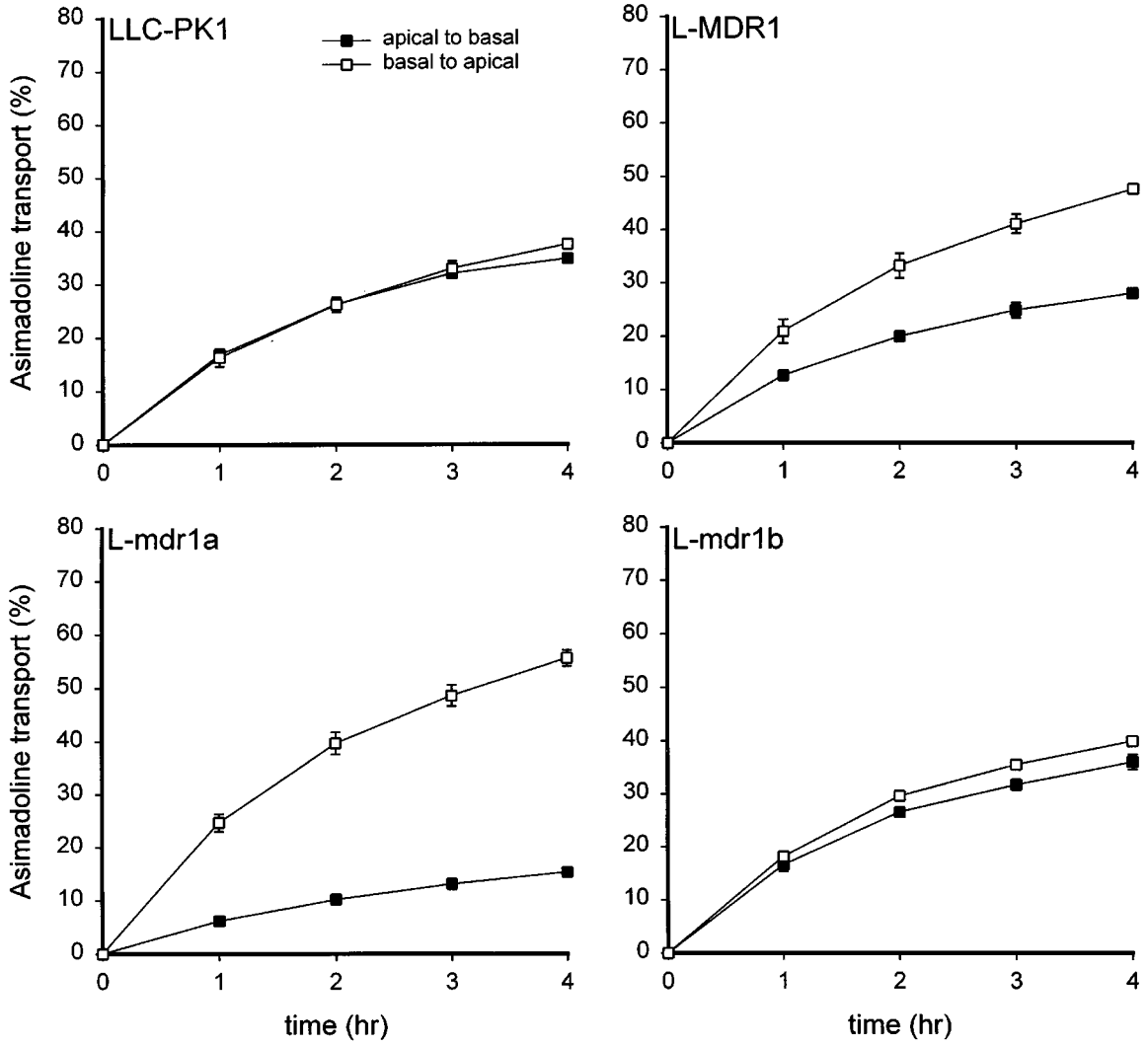

Figure 1 Transepithelial transport of [^{14}C-]-asimadoline (10 μM) in LLC-PK1, L-*mdr1a*, L-*mdr1b* and L-*MDR1* monolayers. At $t=0$, the radioactive drug was applied in one compartment (basal or apical), and the percentage of radioactivity appearing in the opposite compartment at $t=1$, 2, 3 and 4 h was measured and plotted. Data show a representative experiment (with $n=3$) of three independent experiments (each experiment performed at least in duplicate). Results are expressed as mean values, with bars indicating the s.d. (for some values the range is smaller than the size of the symbols used). One per cent translocation per hour corresponds to a permeability coefficient of about 0.042 mm h^{-1}.

(+/+) and (−/−) mice (Figure 2). *Mdr1a/1b* (−/−) mice were at least 8 fold more sensitive to the sedative effect of asimadoline than wild-type mice: clear sedation was observed at 0.31 mg kg^{-1} in *mdr1a/1b* (−/−) mice, whereas wild-type mice needed at least 2.5 mg kg^{-1} to show a demonstrable sedative effect. Moreover, the time span of sedation was always far longer in *mdr1a/1b* (−/−) than in wild-type mice at the same asimadoline dosage.

To determine the minimal concentration of asimadoline in the brain which still elicits sedation, we measured the brain and plasma concentrations of unchanged asimadoline at 40 and 150 min after intravenous injection of 5 mg kg^{-1} [^{14}C]-asimadoline. We chose these time points and this dosage since the sedative effect of asimadoline wears off at 40 and 150 min after administration of 5 mg kg^{-1} asimadoline in wild-type and *mdr1a/1b* (−/−) mice, respectively (Figure 2). Table 2 indicates that at $t=40$ min, the brain concentration of unchanged asimadoline in *mdr1a/1b* (−/−) mice was 8 fold higher than in wild-type mice, whereas plasma levels were roughly comparable in both mouse strains. At $t=150$ min, the brain concentration of asimadoline in *mdr1a/1b* (−/−) mice was 11 fold higher than in wild-type mice, whereas the plasma levels were again not significantly different between the two mouse strains. The absolute level of asimadoline in *mdr1a/1b* (−/−) brain at $t=150$ min was comparable to that in wild-type brain at $t=40$ (357±72 versus 493±110 ng g^{-1}, respectively). This corresponds well with the respective time points at which sedation wears off in both mouse strains, and it indicates that it is the brain level of unchanged asimadoline that determines the degree of sedation in both mouse strains. The data further show that asimadoline is quite rapidly cleared from both wild-type and *mdr1a/1b* (−/−) brain, as evidenced by an 11–14 fold drop in brain concentration between 40 and 150 min.

Comparison with total radioactivity data (not shown) indicated that the fraction of unmetabolized asimadoline in brain and plasma did not differ substantially between wild-type and *mdr1a/1b* (−/−) mice at either 40 or 150 min after administration (in brain: 75–85% at $t=40$ min, and 25–30% at $t=150$ min; in plasma 40–45% at $t=40$ min, and 8–14% at $t=150$ min).

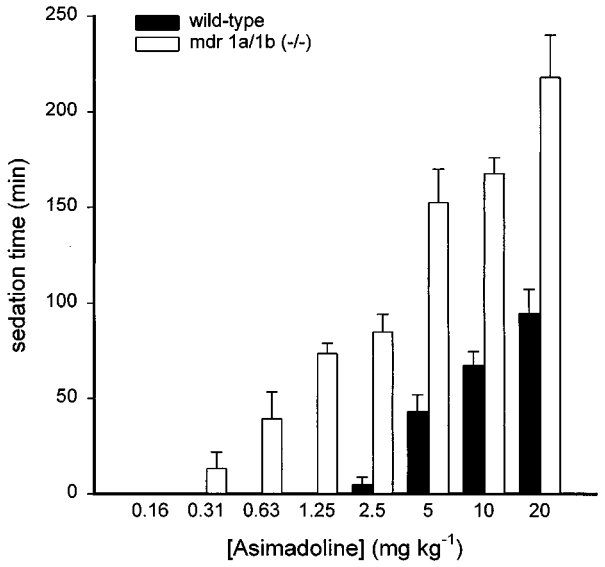

Figure 2 Sedative effects of asimadoline in *mdr1a/1b* (−/−) mice (open columns) and *mdr1a/1b* (+/+) mice (solid columns). Results are expressed as sedation times (means±s.d.) with $n=4$. Asimadoline was administered i.v. at doses of 1.25, 2.5, 5, 10 and 20 mg kg^{-1} for both *mdr1a/1b* (−/−) and *mdr1a/1b* (+/+). Doses below 1.25 mg kg^{-1} were given only to *mdr1a/1b* (−/−) mice.

Table 2 Brain and plasma levels of unchanged asimadoline in *mdr1a/1b* (+/+) and (−/−) mice at 40 and 150 min after intravenous injection with 5 mg kg^{-1} [^{14}C]-asimadoline

	mdr1a/1b (+/+) (ng g^{-1})	mdr1a/1b (−/−) (ng g^{-1})	*Ratio (−/−):(+/+)*
40 min			
Brain	493±110	4054±524	8.2**
Plasma	672±158	610±156	0.9
150 min			
Brain	34±17	357±72†	10.6**
Plasma	88±44	50±15†	0.6

Results are expressed as mean asimadoline concentrations (ng g^{-1})±s.d., $n=6$, †$n=5$; **$P<0.01$.

Excretion of [^{14C}]-asimadoline in mdr1a/1b *(+/+) and (−/−) mice*

We next measured the hepatobiliary, intestinal and urinary excretion of [^{14}C]-asimadoline in animals in which the gallbladder was cannulated. After intravenous injection of 1 mg kg^{-1} [^{14}C]-asimadoline, bile was collected during 1 h. Table 3 shows that radioactivity was mainly excreted *via* the bile in both *mdr1a/1b* (+/+) and (−/−) mice (35–40% of the dose within 1 h). More than 99% of radioactivity in bile was present as metabolite in both wild-type and knockout mice. Excretion of unmetabolized asimadoline in bile, although very low, was about 2 fold higher in wild-type compared to knockout mice (0.47±0.03 and 0.25±0.04%, respectively of total radioactivity in bile ($P<0.001$)). Direct intestinal excretion of radioactivity was found to be about 2 fold reduced in knockout mice, from 5–6% of the dose in (+/+) mice to 3% in (−/−) mice. Urinary excretion was quite variable, but it remained below 1% of the dose in both *mdr1a/1b* (+/+) and (−/−) mice, and was thus negligible. Plasma

Table 3 Excretion and tissue levels of radioactivity in *mdr1a/1b* (+/+) and (−/−) mice with a cannulated gallbladder

Tissue	mdr1a/1b (+/+) (ng-eq g^{-1})	mdr1a/1b (−/−) (ng-eq g^{-1})	*Ratio (−/−):(+/+)*
Brain	114±5	786±153	6.9**
Muscle	316±90	315±106	1.0
Heart	503±123	458±97	0.9
Kidney	1873±179	1848±460	1.0
Liver	4481±443	4523±595	1.0
Lung	3764±597	2911±647	0.8
Spleen	1158±429	1208±668	1.0
Testis	288±144	797±51	2.8**
Plasma	275±27	264±63	1.0
Bile (%)‡	38±16	36±4	1.0
Intestinal cont. (%)†	5.6±1.1	3.0±1.4	0.5*
Urine (%)	0.36±0.45	0.37±0.46	1.0

Tissue levels of radioactivity at 60 min after intravenous injection of 1 mg kg^{-1} [^{14}C]-asimadoline. Results are expressed as mean [^{14}C]-concentrations (ng-eq g^{-1})±s.d., $n=4$; *$P<0.05$, **$P<0.01$. For bile, intestinal contents and for urine, results are expressed as percentage of the dose [^{14}C]-asimadoline; †Represents combined excretion into small intestine, cecum and colon; ‡The amount of bile produced over 1 h was similar in *mdr1a/1b* (+/+) and (−/−) mice.

and tissue levels of radioactivity at $t=60$ min in this experiment were comparable between *mdr1a/1b* (+/+) and (−/−) mice, except for brain and testis.

Oral availability of asimadoline in mdr1a/1b *(+/+) and (−/−) mice*

Comparison of plasma concentrations of unmetabolized asimadoline over time after i.v. and p.o. administration indicated that asimadoline has a bioavailability of only 13% in rats (Barber *et al*., 1994). A low bioavailability of drugs can be the result of active excretion or back-transport of the drugs by P-gp in the intestinal mucosa (Sparreboom *et al*., 1997; Kim *et al*., 1998). Although the contribution of intestinal mdr1a P-gp to direct intestinal excretion of asimadoline was relatively small (Table 3), we decided to test whether the bioavailability of asimadoline is diminished by P-gp by measuring oral uptake of the drug in our knockout model. We compared the plasma and tissue levels of radioactivity in *mdr1a/1b* (+/+) and (−/−) mice which had been given an oral dose of 1 mg kg^{-1} [^{14}C]-asimadoline. The results are shown in Table 4. Plasma and tissue levels of radioactivity after 1 h were comparable (with the expected exception of brain and testis), suggesting that P-gp does not substantially influence the oral uptake of asimadoline. In all animals, 20–30% of radioactivity was still found in the stomach, whereas about 50% of the label was present in the small intestine. Negligible levels were present in cecum and colon. As the remainder of the radioactivity must have been absorbed, the uptake of asimadoline from the gut is quite high, comprising at least 20–30% of the administered dose within 1 h.

Discussion

This study demonstrates that in mice, blood-brain barrier (mdr1a) P-gp is a major determinant for the low brain penetration of asimadoline, and thus for the very limited sedative CNS side-effects of this drug. Since asimadoline is also a transported substrate of the human MDR1 P-gp, the same protective effect will most likely occur in human brain. It thus appears that the specific therapeutic purpose for which asimadoline is developed, *viz*. a peripherally acting analgaesic with very limited sedative and other adverse side-effects in the CNS (Barber *et al*., 1994; Gottschlich *et al*., 1995) is to a considerable extent determined by the presence of P-gp in the blood-brain barrier. Interestingly, in spite of the prominent role of mdr1a P-gp in the blood-brain barrier for this drug, we found that intestinal mdr1a P-gp has relatively little effect on the oral uptake of asimadoline. This indicates that, at least in some cases, it is feasible to develop drugs that are kept out of the brain by P-gp, with therapeutically beneficial effects, whereas at the same time intestinal P-gp is not an unsurmountable barrier for oral administration of the drug.

One characteristic in which asimadoline differs from some other drugs for which we have previously demonstrated a strong effect of blood-brain barrier P-gp is the rate of clearance of the drug from the brain. Whereas vinblastine is cleared very slowly from the brain of *mdr1a* (−/−) (and wild-type) mice, and whereas [^{3}H]-digoxin even demonstrates a gradual accumulation in *mdr1a* (−/−) brain over a period of more than 2 days (Schinkel *et al*., 1994; Mayer *et al*., 1996), asimadoline was quite rapidly cleared from both wild-type and *mdr1a/1b* (−/−) brain (see Table 2). These differences in behaviour between the various drugs may result from the relative tightness of binding to brain components, the degree to which each drug is metabolized in the brain, the presence of export systems other than P-gp for the parent drug and/or their metabolites, or a combination of these properties. Whatever the exact cause of these differences, it is clear that various drugs that are all clearly affected by blood-brain barrier P-gp may still differ widely in their pharmacokinetic behaviour in the brain.

Next to the brain, the relative accumulation of asimadoline in the testis was also markedly (about 3 fold) increased in *mdr1a/1b* (−/−) mice (Tables 1, 3 and 4). Similar effects were previously observed with the drugs ivermectin, cyclosporin A, ondansetron and loperamide in *mdr1a* (−/−) mice, and they are most likely ascribed to the presence of mdr1a P-gp in the luminal membrane of endothelial cells at the blood-testis barrier (Cordon-Cardo *et al*., 1989; Schinkel *et al*., 1994; 1995; 1996). These results further support the idea that the main biological function of mdr1-type P-gp is protection of a range of critical tissues from exogenous xenobiotic toxins (Schinkel, 1997).

Earlier studies have shown that the excretion and oral uptake of drugs transported by P-gp can be extensively affected in *mdr1a* (−/−) and *mdr1a/1b* (−/−) mice (Mayer *et al*., 1996; 1997; Sparreboom *et al*., 1997; Van Asperen *et al*., 1997a; Kim *et al*., 1998). The hepatobiliary excretion of a number of cationic amphiphilic compounds for example was found to be decreased 2–3 fold in *mdr1a* (−/−) mice (Smit *et al*., 1998a), and for several drugs (e.g. digoxin, paclitaxel) a decreased direct intestinal excretion in *mdr1a* (−/−) mice was reported (Mayer *et al*., 1996; 1997; Sparreboom *et al*., 1997). In the case of asimadoline, the biliary excretion of radioactivity (35–40% of the dose over 1 h) was not noticeably altered in *mdr1a/1b* (−/−) mice, and more than 99% of this radioactivity in bile consisted of metabolites, indicating very efficient metabolism of asimadoline in the liver. At the same time, excretion of unchanged asimadoline, as a percentage of total radioactivity excreted in bile, was significantly diminished from 0.47 ± 0.03% in wild-type mice to 0.25 ± 0.04% in knockout mice. These data suggest that unchanged asimadoline, and not its metabolites, are transported by P-gp in the liver. The very high and unaltered level of hepatobiliary excretion of asimadoline metabolites also explains why no clear differences in plasma levels were observed between wild-type and *mdr1a/1b* (−/−) mice up to 150 min after i.v. asimadoline administration (Table 2).

Table 4 Tissue levels of radioactivity in *mdr1a/1b* (+/+) and (−/−) mice at 60 min after oral administration of 1 mg kg^{-1} [^{14}C]-asimadoline

Tissue	mdr1a/1b (+/+) (ng-eq g^{-1})	mdr1a/1b (−/−) (ng-eq g^{-1})	*ratio (−/−):(+/+)*
Brain	19±5	112±11	5.8**
Muscle	68±22	54±14	0.8
Heart	147±84	130±23	0.9
Kidney	498±81	534±130	1.1
Liver	1436±443	1228±370	0.9
Lung	543±206	491±54	0.9
Spleen	173±40	189±34	1.1
Testis	26±5	57±8	2.2**
Plasma	94±21	106±15	1.1
Stomach (%)†	25.1±11.6	26.0±17.6	1.0
Small int. (%)†	54.6±6.0	53.0±22.4	1.0
Cecum (%)†	0.16±0.05	0.08±0.05	0.5**
Colon (%)†	0.08±0.01	0.07±0.01	0.9

Results are expressed as mean [$^{-14}$C]-concentrations (ng-eq g^{-1})±s.d., $n=4$; **$P<0.01$; †Tissue + contents, results are expressed as percentage of the dose [$^{-14}$C]-asimadoline.

The direct intestinal excretion of [^{14}C]-asimadoline decreased from 6 to 3% of the dose. This 2 fold reduction in direct intestinal excretion of asimadoline observed in knockout mice demonstrates that intestinal mdr1a P-gp can transport the drug. Nevertheless, the absence of P-gp from the intestinal mucosa did not noticeably affect the oral availability of asimadoline. We found that 1 h after oral administration, at least 20% of [^{14}C]-asimadoline (dosed at 1 mg kg^{-1}) had already been absorbed from the gastrointestinal tract in both wild-type and *mdr1a/1b* (−/−) mice, and, the plasma levels of the drug were comparable in both mouse strains. Considering the high hepatobiliary excretion of this drug (35–40% of the dose in 1 h after i.v. administration), the total uptake of the drug from the intestine over this period has probably been higher than 20%. In line with this high uptake, it has been found that about 80% of an oral dose of asimadoline was absorbed within 24 h in rats. In contrast to this high uptake, bioavailability is low (13% in rats, Barber *et al*., 1994), which suggests a high first pass metabolism of asimadoline. However this may be, unlike for paclitaxel, for which the oral availability is strongly limited by P-gp in the intestinal mucosa (Sparreboom *et al*., 1997), P-gp does not seem to be important for the rate of uptake of asimadoline from the intestine.

At this moment we do not know why the uptake of some drugs (such as paclitaxel) is strongly affected by mdr1a P-gp in both the intestine and the blood-brain barrier (Van Asperen *et al*., 1997a,b), whereas the uptake of other drugs (such as asimadoline) is only affected by mdr1a P-gp in the blood-brain barrier. It could be that the rate of uptake of orally administered asimadoline through the intestinal wall is just too rapid for P-gp to make a substantial difference in the net uptake rate by back-transport. This high uptake rate may result from rapid transmembrane diffusion, or perhaps from the presence of facilitating carrier uptake systems for asimadoline that are present in the intestine but not in the blood-brain barrier. The presence of paclitaxel metabolizing activity in the intestinal epithelial cells (e.g. cytochrome P450-3A) in combination with a reduced influx of paclitaxel due to P-gp might further boost the limiting effect of P-gp on overall uptake of unchanged drug (Wacher *et al*., 1995). Whatever the mechanisms behind the differences in intestinal uptake of various drugs, it will clearly be of great interest to study them in more detail, as they may be highly relevant to the clinical application of many drugs.

We do not know why the net renal excretion of asimadoline (and its metabolites) is almost negligible in mice. Based on the substantial asimadoline transport observed in the pig-kidney cell line, one might have expected a more substantial contribution of the kidney to asimadoline clearance. Perhaps murine kidney epithelial cells do not efficiently take up asimadoline, or maybe asimadoline is efficiently reabsorbed in a distal part of the kidney tubules. Clearly, as discussed above for oral uptake of asimadoline, much more research will be necessary before we will be able to make reliable predictions of the pharmacokinetic behaviour of drugs transported by P-gp.

In summary, this study adds a new drug to the list of compounds for which the (potential) clinical pharmacological applications are largely determined by the presence of blood-brain barrier P-gp. Other examples include ivermectin, which is used to combat onchocerciasis in humans at dosages that would be close to neurotoxic levels in the absence of blood-brain barrier P-gp (Schinkel *et al*., 1994), domperidone, used as peripherally acting dopamine antagonist anti-emetic, and loperamide, an opiate without CNS side-effects due to P-gp activity, and consequently used as an antidiarrheal drug (Schinkel *et al*., 1996). We expect that further research will reveal many more drugs that can be added to this list.

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References

BARBER, A., BARTOSZYK, G.D., BENDER, H.M., GOTTSCHLICH, R., GREINER, H.E., HARTING, J., MAULER, F., MINCK, K.O., MURRAY, R.D., SIMON, M. & SEYFRIED, C.A. (1994). A pharmacological profile of the novel, peripherally-selective kappa-opioid receptor agonist, EMD 61753. *Br. J. Pharmacol.,* **113,** 1317–1327.

BORST, P. & SCHINKEL, A.H. (1997). Genetic dissection of the function of mammalian P-glycoproteins. *Trends Genet.,* **13,** 217–222.

CHEN, C., CHIN, J.E., UEDA, K., CLARK, D.P., PASTAN, I., GOTTESMAN, M.M. & RONINSON, I.B. (1986). Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells. *Cell,* **47,** 381–389.

CORDON-CARDO, C., O'BRIEN, J.P., CASALS, D., RITTMAN-GRAUER, L., BIEDLER, J.L., MELAMED, M.R. & BERTINO, J.R. (1989). Multidrug resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc. Natl. Acad. Sci. U.S.A.,* **86,** 695–698.

DRION, N., LEMAIRE, M., LEFAUCONNIER, J.M. & SCHERRMANN, J.M. (1996). Role of P-glycoprotein in the blood-brain transport of colchicine and vinblastine. *J. Neurochem.,* **67,** 1688–1693.

ENDICOTT, J.A. & LING, V. (1989). The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu. Rev. Biochem.,* **58,** 137–171.

FRICKER, G., DREWE, J., HUWYLER, J., GUTMANN, H. & BEGLINGER, C. (1996). Relevance of p-glycoprotein for the enteral absorption of cyclosporin A: in vitro-in vivo correlation. *Br. J. Pharmacol.,* **118,** 1841–1847.

GOTTESMAN, M.M. & PASTAN, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.,* **62,** 385–427.

GOTTSCHLICH, R., BARBER, A., BARTOSZYK, G.D. & SEYFRIED, C.A. (1995). The peripherally acting κ-opiate agonist EMD 61753 and analogues: opioid activity versus peripheral selectivity. *Drugs Exptl. Clin. Res.,* **21,** 171–174.

GROS, P., CROOP, J. & HOUSMAN, D. (1986). Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. *Cell,* **47,** 371–380.

HUWYLER, J., DREWE, J., KLUSEMANN, C. & FRICKER, G. (1996). Evidence for P-glycoprotein-modulated penetration of morphine-6-glucuronide into brain capillary endothelium. *Br. J. Pharmacol.,* **118,** 1879–1885.

JULIANO, R.L. & LING, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta,* **445,** 152–162.

KIM, R.B., FROMM, M.F., WANDEL, C., LEAKE, B., WOOD, A.J.J. & RODEN, D.M. (1998). The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. *J. Clin. Invest.,* **101,** 289–294.

MAYER, U., WAGENAAR, E., BEIJNEN, J.H., SMITH, J.W., MEIJER, D.K.F., VAN ASPEREN, J., BORST, P. & SCHINKEL, A.H. (1996). Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr1a P-glycoprotein. *Br. J. Pharmacol.,* **119,** 1038–1044.

MAYER, U., WAGENAAR, E., DOROBEK, B., BEIJNEN, J.H., BORST, P. & SCHINKEL, A.H. (1997). Full blockade of intestinal P-glycoprotein and extensive inhibition of blood-brain barrier P-glycoprotein by oral treatment of mice with PSC833. *J. Clin. Invest.,* **100,** 2430 – 2436.

SCHINKEL, A.H. (1997). The physiological function of drug-transporting P-glycoproteins. *Semin. Cancer Biol.,* **8,** 161 – 170.

SCHINKEL, A.H., MAYER, U., WAGENAAR, E., MOL, C.A.A.M., VAN DEEMTER, L., SMIT, J.J.M., VAN DER VALK, M.A., VOORDOUW, A.C., SPITS, H., VAN TELLINGEN, O., ZIJLMANS, J.M.J.M., FIBBE, W.E. & BORST, P. (1997). Normal viability and altered phamacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 4028 – 4033.

SCHINKEL, A.H., SMIT, J.J.M., VAN TELLINGEN, O., BEIJNEN, J.H., WAGENAAR, E., VAN DEEMTER, L., MOL, C.A.A.M., VAN DER VALK, M.A., ROBANUS-MAANDAG, E.C., TE RIELE, H.P.J., BERNS, A.J.M. & BORST, P. (1994). Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell,* **77,** 491 – 502.

SCHINKEL, A.H., WAGENAAR, E., MOL, C.A.A.M. & VAN DEEMTER, L. (1996). P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *J. Clin. Invest.,* **97,** 2517 – 2524.

SCHINKEL, A.H., WAGENAAR, E., VAN DEEMTER, L., MOL, C.A.A.M. & BORST, P. (1995). Absence of the mdr1a P-glycoprotein in mice affects tissue distribution and pharmacokinetics of Dexamethasone, Digoxin and Cyclosporin A. *J. Clin. Invest.,* **96,** 1698 – 1705.

SMIT, J.W., SCHINKEL, A.H., MÜLLER, M., WEERT, B. & MEIJER, D.K.F. (1998a). Contribution of the murine mdr1a P-glycoprotein to hepatobiliary and intestinal elimination of cationic drugs as measured in mice with an *mdr1a* gene disruption. *Hepatology,* **27,** 1056 – 1063.

SMIT, J.W., WEERT, B., SCHINKEL, A.H. & MEIJER, D.K.F. (1998b). Heterologous expression of various P-glycoproteins in polarized epithelial cells induces directional transport of small (type 1) and bulky (type 2) cationic drugs. *J. Pharmacol. Exp. Ther.,* **286,** 321 – 327.

SPARREBOOM, A., VAN ASPEREN, J., MAYER, U., SCHINKEL, A.H., SMIT, J.W., MEIJER, D.K.F., BORST, P., NOOIJEN, W.J., BEIJNEN, J. & VAN TELLINGEN, O. (1997). Limited oral bioavailability and active epithelial excretion of paclitaxel (taxol) caused by P-glycoprotein in the intestine. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 2031 – 2035.

STEIN, C. & SCHÄFER, M. (1997). Peripheral opioid analgesia: basic and clinical aspects. *Semin. Anesth.,* **16,** 112 – 116.

VAN ASPEREN, J., MAYER, U., VAN TELLINGEN, O. & BEIJNEN, J.H. (1997a). The functional role of P-glycoprotein in the blood-brain barrier. *J. Pharm. Sci.,* **86,** 881 – 884.

VAN ASPEREN, J., VAN TELLINGEN, O., SPARREBOOM, A., SCHINKEL, A.H., BORST, P., NOOIJEN, W.J. & BEIJNEN, J.H. (1997b). Enhanced oral bioavailability of paclitaxel in mice treated with the P-glycoprotein blocker SDZ PSC 833. *Br. J. Cancer,* **76,** 1181 – 1183.

WACHER, V.J., WU, C.-Y. & BENET, L.Z. (1995). Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. *Mol. Carcinogen.,* **13,** 129 – 134.

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