JPP Journal of Pharmacy and Pharmacology

JPP 2002, 54: 1055–1063

Received September 21, 2001
Accepted April 9, 2002
ISSN 0022-3573

Contribution of P-glycoprotein to efflux of ramosetron, a 5-HT_3 receptor antagonist, across the blood–brain barrier

Chika Yamamoto, Hideyasu Murakami, Noriko Koyabu, Hitomi Takanaga, Hirotami Matsuo, Takeshi Uchiumi, Michihiko Kuwano, Mikihiko Naito, Takashi Tsuruo, Hisakazu Ohtani and Yasufumi Sawada

Abstract

In-situ rat and mouse brain perfusion data indicated that the brain distribution of ramosetron (*R*-ramosetron), a 5-hydroxytryptamine$_3$ (5-HT_3) receptor antagonist, was extremely low compared with that expected from its lipophilicity. We hypothesized the involvement of an efflux system(s) and investigated the contribution of P-glycoprotein to efflux transport of ramosetron across the blood–brain barrier by means of an in-vitro uptake study in cell lines that over-express P-glycoprotein. We examined the contributions of *mdr1a*, *mdr1b* and *MDR1* P-glycoprotein by using LV500 cells, MBEC4 cells and LLC-GA5-COL300 cells, which over-express *mdr1a* P-glycoprotein, *mdr1b* P-glycoprotein and *MDR1* P-glycoprotein, respectively. The uptake of [^{14}C]ramosetron by LV500 cells and LLC-GA5-COL300 cells was significantly lower than that by the respective parental cells. Next, we studied the effects of P-glycoprotein inhibitors, verapamil and ciclosporin, on uptake of [^{14}C]ramosetron by these cell lines. The uptake of [^{14}C]ramosetron by LV500 cells and LLC-GA5-COL300 cells was significantly increased in the presence of verapamil or ciclosporin, while verapamil did not affect the uptake of [^{14}C]ramosetron by MBEC4 cells. These results indicate that the efflux of [^{14}C]ramosetron is partly mediated by *mdr1a* P-glycoprotein, but not by *mdr1b* P-glycoprotein, and that there is a difference in substrate specificity between *mdr1a* P-glycoprotein and *mdr1b* P-glycoprotein. Further, [^{14}C]ramosetron was confirmed to be effluxed by human *MDR1* P-glycoprotein. We conclude that the limited distribution of ramosetron to the brain is due, at least in part, to efflux mediated by the P-glycoprotein at the blood–brain barrier.

Department of Medico-Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Chika Yamamoto, Hideyasu Murakami, Noriko Koyabu, Hitomi Takanaga, Hirotami Matsuo, Hisakazu Ohtani, Yasufumi Sawada

Department of Medical Biochemistry, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Takeshi Uchiumi, Michihiko Kuwano

Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

Mikihiko Naito, Takashi Tsuruo

Correspondence: Y. Sawada, Department of Medico-Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan. E-mail: sawada@phar.kyushu-u.ac.jp

Introduction

Ramosetron, a serotonin 5-hydroxytryptamine$_3$ (5-HT_3) receptor-selective antagonist, is a potent antiemetic. It acts at 5-HT_3 receptors located in small intestinal myenteric afferent vagal neural terminals to suppress nausea and vomiting induced by serotonin, which is released when anticancer agents (cisplatin, etc.) accumulate in the mucosa of the digestive tract and act on mucosal chromaffin cells (EC cells). Ramosetron is more effective than other 5-HT_3 antagonists (granisetron, ondansetron, etc.) in terms of inhibitory potency, binding rate constant and pharmacological activity. Moreover, the distribution of ramosetron to the brain in rats was much lower than that to other organs such as liver, intestine, lung and kidney, despite its high lipophilicity (Nakamura et al 1994). The cerebrum-to-plasma concentration ratio was 0.17 at 5 min after intravenous administration (Nakamura et al 1994). These observations might be explained in terms of a specific mechanism restricting its entry into the brain.

Drug efflux mediators, such as P-glycoprotein, are present at the blood–brain barrier, where they are important factors controlling drug distribution to the CNS (Cordon et al 1989; Tsuji et al 1992; Schinkel et al 1994, 1996). P-Glycoprotein was originally identified as a 170-kDa glycoprotein that confers multidrug resistance upon tumour cells (Juliano & Ling 1976). It is present at the luminal surface of normal tissues such as brain, intestine, adrenal gland, pancreas, kidney and liver (Thiebaut et al 1987; Tsuji &

Tamai 1997), and may play a physiological role in the excretion from the cells of hormones and endogenous compounds. It also functions as an efflux pump for xenobiotics and their metabolites. P-glycoprotein recognizes a variety of substrates and (or) inhibitors including cytotoxic agents (*Vinca* alkaloids, anthracyclines, epipodophyllotoxins, dactinomycin, etc.), immunosuppressants (ciclosporin, tacrolimus), calcium-channel blockers (diltiazem, nicardipine, verapamil), beta blockers (acebutolol, celiprolol, nadolol, timolol, bunitrolol) and 5-HT_3 antagonists (azasetron, ondansetron). Thus, since it recognizes diverse substrates, P-glycoprotein has a significant influence on the pharmacokinetics and interactions of many drugs.

P-glycoproteins are encoded by the *MDR* gene family, which consists of two members in man (*MDR1* and *MDR3*) and three members in mice (*mdr1a*, *mdr1b* and *mdr2*) (Hsu et al 1989; Devault & Gros 1990; Lincke et al 1991). The human *MDR1* P-glycoprotein is present in kidney, liver, intestine and brain, while mouse *mdr1a* P-glycoprotein is present in intestine, liver and brain, and mouse *mdr1b* P-glycoprotein is present in adrenal gland, placenta, ovary and uterus (Thiebaut et al 1987; Cordon et al 1989; Croop et al 1989; Borst et al 1993; Schinkel et al 1994). Although *mdr1a* P-glycoprotein and *mdr1b* P-glycoprotein recognize similar substrates (Gros et al 1991), their drug-transporting abilities are different (Cohen et al 1990; Yang et al 1990). For instance, it was reported that progesterone inhibited drug binding and efflux and increased sensitivity to vinblastine with more potency in J7. V1-1 cells, which over-express *mdr1b* P-glycprotein, than in J7. V3-1 cells, that over-express *mdr1a* P-glycoprotein. Previously, we found that bunitrolol is a substrate for *mdr1a* P-glycoprotein, but is poorly recognized by *mdr1b* P-glycoprotein (Matsuzaki et al 1999). In practice, animals such as rats and mice are generally used to investigate drug kinetics, and the data are extrapolated to man. To establish the validity, or otherwise, of this approach, we need to determine the precise substrate specificities and the expression sites of *mdr1a* P-glycoprotein and *mdr1b* P-glycoprotein.

In this study, we hypothesized that the limited distribution of ramosetron into the brain is due to the existence of an efflux system(s), based on the results of in-situ rat and mouse brain perfusion, and we investigated the contributions of *MDR1* P-glycoprotein, *mdr1a* P-glycoprotein, and *mdr1b* P-glycoprotein to the cellular accumulation of ramosetron by means of uptake studies in-vitro, using cell lines over-expressing each P-glycoprotein.

Materials and Methods

Materials and animals

[^{14}C]Ramosetron (1.26 GBq mmol^{-1}) and ramosetron were kindly supplied by Yamanouchi Pharmaceutical Co. Ltd (Tokyo, Japan). [^{14}C]Ramosetron was purified before use as described below. 4-[*N*-Methyl-^{14}C]iodoantipyrine (2.5 Gbq mmol^{-1}) was purchased from American Radiolabeled Chemicals, Inc. (MO). [^{3}H]Vinblastine (529 GBq mmol^{-1}) and DL-[4-^{3}H]propranolol hydrochloride (1.04 Tbq mmol^{-1}) were purchased from Amersham International (Buckinghamshire, UK). [Benzene ring-^{3}H(N)] imipramine hydrochloride (1.9 Tbq mmol^{-1}), [^{3}H]inulin (2.6 mCi g^{-1}) and [fructose-1-^{3}H(N)]sucrose (455.1 Gbq mmol^{-1}) were purchased from New England Nuclear Co. (MA). Verapamil hydrochloride was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were commercial products of reagent grade.

Male ddY mice (20–25 g) and male Wistar rats (170–230 g) were purchased from Seac Yoshitomi, Ltd. (Fukuoka, Japan). The mice and rats were housed under controlled environmental conditions (general food and water were freely available) and treated according to the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

Purification of [^{14}C]ramosetron

A solution of [^{14}C]ramosetron in ethanol was purified by thin-layer chromatography on silica gel in chloroform–methanol–water (12:6:1) using non-radiolabelled ramosetron as the standard. The single spot of unchanged [^{14}C]ramosetron was scraped from the plate after UV detection and a saturated sodium bicarbonate solution was added. The solution was shaken and left for 5 min at room temperature. Ethyl acetate was added and the solution was shaken and then centrifuged for 30 s at 800 *g*. The organic layer was transferred to another centrifuge tube and dried up under an N_2 gas flow. Radioactivity of [^{14}C]ramosetron was measured with a liquid scintillation counter (LS6500, Beckman Instrument, Inc., CA) after the residue had been dissolved in ethanol and scintillation fluid added (Clearsol I, Nacalai Tesque Inc., Kyoto, Japan). Samples were stored at -20°C until required.

In-situ brain perfusion

The right cerebral hemisphere of rat or mouse was perfused by the method reported previously (Takasato et al 1984; Murakami et al 2000). In brief, adult male rats (Wistar, 170–230 g body weight) or adult male mice (ddY, 20–25 g body weight) were anaesthetized with pentobarbital sodium (50 mg kg^{-1}, i.p.) (Dainippon Pharmaceutical Co. Ltd, Osaka, Japan). The right carotid arteries were exposed, the occipital and superior thyroid arteries were coagulated and cut, and the right pterygopalatine artery was ligated. Then, the right external carotid artery was catheterized for retrograde infusion with 10 cm of polyethylene tubing (SP-31 (rat) or SP-10 (mouse)) (Natume Seisakusho, Tokyo, Japan) filled with heparinized saline. The opening of the catheter was placed 4–5 mm (rat) or 2–3 mm (mouse) above the bifurcation of the common carotid artery. The right common carotid artery was prepared for ligation by encircling the artery with surgical thread.

Krebs–Henseleit buffer, consisting of (in mM) 118.0 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$ and 10 D-glucose, was oxygenated for 10 min with 95% O_2–5% CO_2 before use as a perfusate. The perfusate

was adjusted to pH 7.4 with 1 M HCl. The perfusion fluid containing radiolabelled drug was infused into the right external carotid artery at a rate of 4.98 mL min^{-1} (rat) or 1 mL min^{-1} (mouse) by an infusion pump (55–1111, Harvard Apparatus, MA) and the right common carotid artery was ligated immediately. Perfusion was continued for 30 s and terminated by decapitation. The right cerebral hemisphere was immediately excised, weighed and placed in a scintillation vial. Samples were digested at 80°C in 1.5 mL of Solvable (Packard, CT), treated with 200 μL of 30% H_2O_2, neutralized by addition of 100 μL of 6 M HCl and prepared for scintillation counting by the addition of scintillation fluid. After decapitation, a 20-μL sample of perfusate was placed in a scintillation vial and prepared for scintillation counting by addition of 15 mL of scintillation fluid. The radioactivity of the brain or perfusate samples was measured with a liquid scintillation counter.

In the perfusion study, blood-to-brain uptake of a drug is given by equation 1 (brain-to-blood back diffusion included) or equation 2 (back diffusion ignored), where Kp (mL (g brain)$^{-1}$) is the ratio of the parenchymal brain concentration of tracer (pmol (g brain)$^{-1}$) to the perfusion fluid concentration (pmol mL^{-1}), t is the perfusion time (s), K1 is influx clearance (mL (g brain)$^{-1}$ s^{-1}) and k2 is the efflux rate constant (s^{-1}).

$$dKp/dt = K1 - k2 \times Kp \quad (1)$$

$$dKp/dt = K1 \quad (2)$$

To determine K1 and k2 of tracer, equation 1 or equation 2 was fitted to the data by means of a nonlinear least-squares procedure (MULTI) (Yamaoka et al 1981) and the equation that gave the lower AIC (Akaike's information criterion) (Yamaoka et al 1981) was adopted. The permeability coefficient–surface area product (PS value) (mL (g brain)$^{-1}$ s^{-1}), which represents the blood-to-brain influx permeability across the capillary wall, was calculated from equation 3 (Takasato et al 1984), where Fpf is the cerebral perfusion fluid flow (mL (g brain)$^{-1}$ s^{-1}).

$$PS = -Fpf \times \ln(1 - K1/Fpf) \quad (3)$$

Fpf values of rat and mouse, which were calculated based on the parenchymal brain uptake of 0.77 μM [^{14}C]diazepam at 5 s, were taken from our previous report (Murakami et al 2000) and were 0.129 mL g^{-1} s^{-1} and 0.071 mL g^{-1} s^{-1}, respectively.

To confirm that the paracellular permeability of the blood–brain barrier is not altered during the experiment, the distribution volume of ^{3}H- or ^{14}C-labelled sucrose, which reflects the intravascular volume, was measured.

Measurement of the octanol/water partition coefficient

n-Octanol and 0.1 mM phosphate buffer (pH 7.4) were used as the organic and aqueous phases, respectively. The initial concentrations of ^{3}H-labelled and ^{14}C-labelled compounds in the aqueous phases were 333 nCi mL^{-1} and 16.7 nCi mL^{-1}, respectively. A sample of drug-containing aqueous phase was mixed with an equal volume of octanol and shaken vigorously for 1 h at 37°C. Then the organic and aqueous phases were separated by centrifugation at 738 *g* for 5 min. The organic and aqueous phases were each placed in a scintillation vial and prepared for scintillation counting by addition of scintillation fluid. The radioactivity was measured with a liquid scintillation counter.

Cell culture

Mouse L cells and LV500 cells were grown in Eagle's minimum essential medium (MEM) (Nissui Seiyaku Co., Tokyo, Japan) containing 10% fetal calf serum (FCS), 0.292 mg mL^{-1} L-glutamine, 100 μg mL^{-1} kanamycin and 100 U mL^{-1} penicillin G (Kusaba et al 1995) in a humidified atmosphere of 5% CO_2–95% air at 37°C. LV500 cells were established by continuous exposure of L cells to 500 ng mL^{-1} vincristine. For the uptake studies, L and LV500 cells were seeded at 4×10^4 cells/well in 4-well multidishes (1.9 cm^2) (Nunc, Denmark), and cultured for 2 days.

Mouse brain capillary endothelial cells (MBEC4 cells) were grown in Dulbecco's modified Eagle's medium (Nikken Bio Medical Laboratory, Kyoto, Japan) containing 10% FCS, 100 μg mL^{-1} streptomycin and 100 U mL^{-1} penicillin G (Tatsuta et al 1992, 1994) in a humidified atmosphere of 5% CO_2–95% air at 37°C. For the uptake studies, MBEC4 cells were seeded at 4×10^4 cells/well in multidishes and cultured for 3 days.

The porcine kidney epithelial cell line, LLC-PK1 and LLC-GA5-COL300 cells were obtained from the Riken Cell Bank (Ibaraki, Japan). LLC-PK1 and LLC-GA5-COL300 cells were grown in medium 199 (Nikken Bio Medical Laboratory, Kyoto, Japan) containing 10% FCS, 100 μg mL^{-1} streptomycin and 100 U mL^{-1} penicillin G (Tanigawara et al 1992; Ueda et al 1992) in a humidified atmosphere of 5% CO_2–95% air at 37°C. LLC-GA5-COL300 cells were grown in the presence of 300 ng mL^{-1} colchicine. For the uptake studies, LLC-PK1 and LLC-GA5-COL300 cells were seeded in multidishes at 1×10^4 and 1.2×10^4 cells/well, respectively, and cultured for 4 days.

Uptake studies

The culture medium was removed and the cells were washed three times with 1.0 mL of incubation buffer (composition (mM): 141 NaCl, 4 KCl, 2.8 $CaCl_2$, 1 $MgSO_4$, 10 D-glucose, 10 HEPES, pH 7.4). We used the same incubation buffer in the uptake study in all the cell lines. The uptake study was initiated by adding 250 μL to multidishes of incubation buffer containing [^{14}C]ramosetron (10 μM) or [^{3}H]vinblastine (30 nM) in the absence or presence of verapamil (10, 50 and 100 μM) or ciclosporin (20 μM). The cells were incubated at 37°C for a specified time. After incubation, the cells were washed three times with 1.0 mL of ice-cold incubation buffer to terminate the uptake. The cells were solubilized with 3 M NaOH, and neutralized with 6 M HCl. [^{14}C]ramosetron or [^{3}H]vinblastine was measured by liquid scintillation counting after addition of scintillation fluid. The same amount of dimethyl sulfoxide (DMSO) was added to the incubation buffer in all experiments, since

verapamil and ciclosporin each had to be dissolved in DMSO. The uptake of test compound by the cells was presented as the ratio of radioactivity in the sample to the concentration in the medium (cell/medium ratio; μL (mg protein)$^{-1}$). The cell/medium ratio is obtained by dividing the uptake amount per the cellular protein amount (pmol (mg protein)$^{-1}$) by the initial concentration in the uptake buffer (μmol L^{-1}). Normalization of the uptake by using the cell/medium ratio is convenient to assess and compare the uptakes of different substrates at different concentrations. Cellular protein was measured by the method of Lowry et al (1951) using bovine serum albumin as the standard.

Statistical analysis

Data were expressed as mean ± s.d. and statistical analysis was performed by using Student's *t*-test or analysis of variance followed by Dunnett's test. A difference between means was considered to be significant when the *P* value was less than 0.05. The number of replicates for each experiment is given in the legend of each figure or table.

Results

In-situ brain perfusion

Figure 1 shows the time courses of the apparent ratio of the concentration of the tracer in the parenchymal brain to that in the perfusion fluid (Kp) for [^{14}C]ramosetron, [^{3}H]propranolol and [^{3}H]imipramine in rats. The Kp value of [^{14}C]ramosetron increased linearly with time up to 30 s. For mouse, the permeability coefficient–surface area (PS) value of [^{14}C]ramosetron was calculated from equation 2 and equation 3 by using the data at 30 s. The data for iodoantipyrine in mouse and rat were taken from our previous report (Murakami et al 2000). The PS values are plotted against the values of octanol/water partition coefficient divided by the square root of the molecular weight in Figure 2. The line in Figure 2 was calculated by the least-squares method based on Takasato's data of cerebrovascular permeability, octanol/water partition coefficients and molecular weights of sucrose, mannitol, glycerol, urea, thiourea, antipyrine, ethyleneglycol and trimethyleneglycol (Takasato et al 1984). The PS values of [^{14}C]iodoantipyrine, [^{3}H]propranolol and [^{3}H]imipramine lay on the line. On the other hand, that of [^{14}C]ramosetron was lower than the predicted value based on the octanol/water partition coefficient and molecular weight.

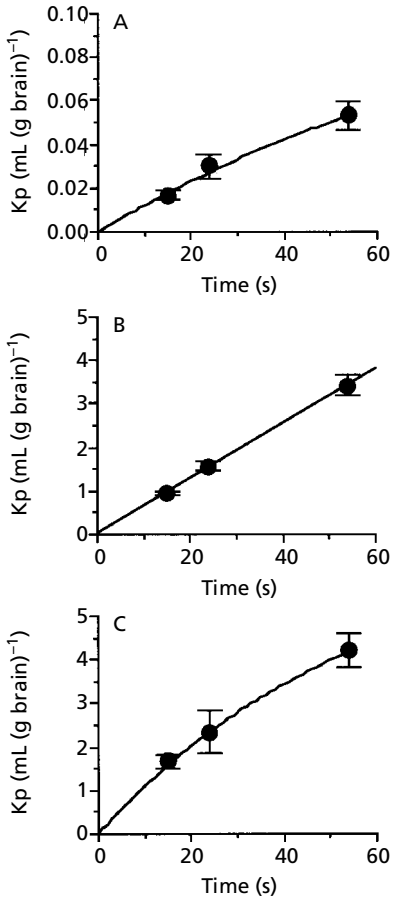

Figure 1 Time courses of the apparent ratio of the parenchymal brain concentration of the tracer to the concentration of the tracer in the perfusion fluid (Kp) for ramosetron (A), propranolol (B) and imipramine (C) in rats. Data are means ± s.d. (n = 3). The lines show the values of the Kp simulated by equation 2 (straight line), which was applied for propranolol (negligible back diffusion), or equation 1 (curved line), which was applied for ramosetron and imipramine (obvious back diffusion).

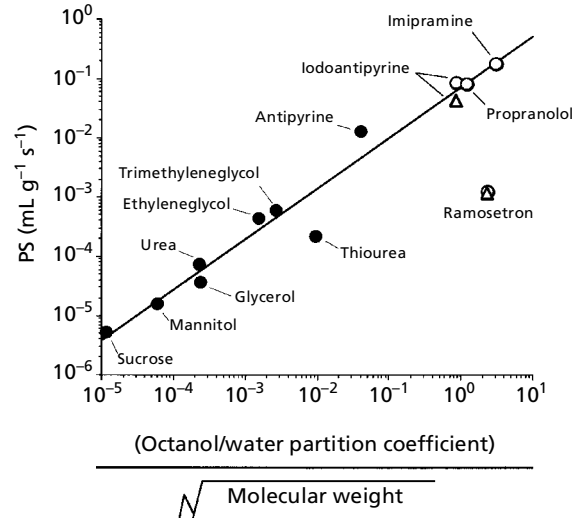

Figure 2 Comparison of the experimental permeability coefficient–surface area (PS) values at the blood–brain barrier with the predicted values based on the octanol/water partition coefficient divided by the square-root of the molecular weight. The line was obtained by least-squares fitting to Takasato's data (Takasato et al 1984), shown by closed circles. Open circles and triangles represent our experimental values obtained by using in-situ rat and mouse brain perfusion, respectively. The data for iodoantipyrine in mouse and rat were taken from our previous report (Murakami et al 2000).

Table 1 Effects of unlabelled ramosetron and verapamil on vascular volume and permeability coefficient–surface area (PS) products of [^{14}C]ramosetron (10 μM) obtained by using the in-situ mouse brain perfusion technique.

	Vascular volume (μL (g brain)$^{-1}$)	PS products of [^{14}C] ramosetron (μL (g brain)$^{-1}$ s^{-1})
Control	11.19±1.68	1.18±0.31
+500 μM Ramosetron	11.34±0.31	3.27±0.12*
+500 μM Verapamil	11.97±0.10	3.12±0.33*

Estimates of PS values in the presence and absence of inhibitors were based on the Kp values at 30 s. Each value represents the mean±s.d. of three experiments. *$P > 0.05$, vs control (analysis of variance followed by Dunnett's test).

Effect of P-glycoprotein inhibitor on transport of [^{14}C]ramosetron at the blood–brain barrier

Table 1 presents the effects of verapamil (500 μM) as a P-glycoprotein inhibitor and a high concentration of unlabelled ramosetron (500 μM) on the transport of [^{14}C] ramosetron in the in-situ mouse brain perfusion model. The PS values of [^{14}C]ramosetron were increased in the presence of unlabelled ramosetron or verapamil. The distribution volume of [^{3}H]sucrose, an index of paracellular permeability, was not affected by unlabelled ramosetron (500 μM) or verapamil (500 μM) (Table 1).

The uptake of [^{3}H]vinblastine and [^{14}C]ramosetron by L and LV500 cells

To investigate the contribution of *mdr1a* P-glycoprotein, which is especially important for P-glycoprotein-mediated function at the blood–brain barrier in mice (Croop et al 1989; Schinkel et al 1994), we carried out uptake experiments using L cells and LV500 cells which over-express *mdr1a* P-glycoprotein (Kusaba et al 1995). The uptake of [^{3}H]vinblastine by LV500 cells was significantly lower than that by L cells, as was the case with the uptake of [^{14}C] ramosetron (Table 2).

Effect of P-glycoprotein inhibitor on uptake of [^{3}H]vinblastine and [^{14}C]ramosetron by LV500 cells

The effect of verapamil on the uptake of [^{3}H]vinblastine (30 nM) and [^{14}C]ramosetron (10 μM) by LV500 cells was examined. The steady-state intracellular accumulation of [^{3}H]vinblastine was significantly increased in the presence of verapamil (10 μM) as an inhibitor of P-glycoprotein (Table 3). The uptake of [^{14}C]ramosetron by LV500 cells was also increased by verapamil (10 μM).

Effect of P-glycoprotein inhibitor on uptake of [^{3}H]vinblastine and [^{14}C]ramosetron by MBEC4 cells

We studied the participation of P-glycoprotein in [^{14}C] ramosetron transport by using MBEC4 cells expressing *mdr1b* P-glycoprotein (Tatsuta et al 1994). In view of the result that uptake of [^{14}C]ramosetron by MBEC4 cells was unchanged in the presence of verapamil (10 μM), we further investigated whether verapamil or ciclosporin influenced the uptake of [^{3}H]vinblastine or [^{14}C]ramosetron by MBEC4 cells. The uptake of [^{3}H]vinblastine (30 nM) was significantly increased in the presence of verapamil (10, 50, 100 μM) or ciclosporin (20 μM) (Table 3), whereas that of [^{14}C]ramosetron (10 μM) was unchanged in the presence of verapamil (10, 50, 100 μM) (Table 3). The uptake of [^{14}C] ramosetron was significantly increased in the presence of ciclosporin (20 μM) (Table 3).

The uptake of [^{3}H]vinblastine and [^{14}C]ramosetron by LLC-PK1 and LLC-GA5-COL300 cells

To evaluate the contribution of human *MDR1* P-glycoprotein, we conducted uptake studies using LLC-PK1 cells and LLC-GA5-COL300 cells, the latter of which is transfected with human *MDR1* cDNA and over-expresses *MDR1* P-glycoprotein (Tanigawara et al 1992; Ueda et al 1992). The uptake of [^{3}H]vinblastine (30 nM) by LLC-GA5-COL300 cells was significantly lower than that by LLC-PK1 cells (Table 2). Similarly, the uptake of [^{14}C]

Table 2 The uptake of [^{3}H]vinblastine and [^{14}C]ramosetron by P-glycoprotein-expressing cell lines.

	Cell/medium ratio at 90 min (μL (mg protein)$^{-1}$)			
	L	LV500	LLC-PK1	LLC-GA5-COL300
Vinblastine	127.00±4.80	85.20±4.80*	172.00±12.60	52.40±5.21*
Ramosetron	36.70±4.30	24.00±3.04*	63.70±3.39	38.30±4.95*

The uptake of [^{3}H]vinblastine (30 nM) and [^{14}C]ramosetron (10 μM) by mouse L cells, LV500 cells over-expressing *mdr1a* P-glycoprotein, a porcine kidney epithelial cell line, LLC-PK1 and LLC-GA5-COL300 cells over-expressing *MDR1* P-glycoprotein was measured at 90 min. Each point represents the mean±s.d. of four experiments. *$P < 0.05$, vs the uptake by parental cells (Student's *t*-test).

Table 3 Inhibitory effects of verapamil and ciclosporin on the [^{3}H]vinblastine and [^{14}C]ramosetron uptake by P-glycoprotein-expressing cell lines.

	Cell/medium ratio (% of control)							
	LV500 cells		MBEC4 cells		LLC-PK1 cells		LLC-GA5-COL300 cells	
	Vinblastine	Ramosetron	Vinblastine	Ramosetron	Vinblastine	Ramosetron	Vinblastine	Ramosetron
Control	100.0±10.8	100.00±5.36	100.00±4.48	100.00±5.98	100.00±2.68	100.00±2.98	100.0±27.8	100±12.6
+10 μM Verapamil	180.0±20.6	129.00±6.02*	310.00±2.38*	105.00±2.98	262.0±27.4*	120.00±8.78*	187.0±31.4*	120.0±12.2*
+50 μM Verapamil	n.d.	n.d.	307.00±3.58*	91.20±4.48	262.0±11.1*	106.00±4.48	276.0±22.2*	164±9.48*
+100 μM Verapamil	n.d.	n.d.	281.00±2.98*	95.50±2.98	277.0±20.4*	91.40±2.88	449.0±40.8*	209±13.7*
+20 μM Ciclosporin	n.d.	n.d.	418.0±14.9*	181.00±5.98*	271.0±30.6*	116.0±7.4*	525.0±64.8*	287±42.4*

The uptake of drugs by LV500 cells was measured at 90 min. Each value represents the mean±s.d. of four experiments. *$P < 0.05$, vs control (Student's *t*-test). The uptake of drugs by MBEC4 cells was measured at 60 min. Each value represents the mean±s.d. of four experiments. *$P < 0.05$, vs control (analysis of variance followed by Dunnett's test). The uptake of drugs by LLC-PK1 cells or LLC-GA5COL300 cells was measured at 90 min. Each value represents the mean±s.d. of four experiments. *$P < 0.05$, vs control (analysis of variance followed by Dunnett's test). n.d., not determined.

ramosetron (10 μM) by LLC-GA5-COL300 cells was significantly lower than that by LLC-PK1 cells (Table 2).

Effects of P-glycoprotein inhibitors on steady-state uptake of [^{3}H]vinblastine and [^{14}C]ramosetron by LLC-PK1 and LLC-GA5-COL300 cells

The effects of verapamil and ciclosporin on steady-state uptake of [^{3}H]vinblastine (30 nM) and [^{14}C]ramosetron (10 μM) by LLC cell lines were examined. The uptake of [^{3}H]vinblastine by LLC cell lines was increased in the presence of verapamil (10, 50, 100 μM) or ciclosporin (20 μM). The ratio of increase was higher in LLC-GA5-COL300 cells than in LLC-PK1 cells (Table 3). The [^{14}C]ramosetron uptake by LLC cell lines was also increased in the presence of verapamil (10, 50, 100 μM) or ciclosporin (20 μM) (Table 3).

Discussion

To examine whether the limited distribution of ramosetron into the brain is due to the existence of an efflux system(s), we carried out in-situ brain perfusion studies and made in-vitro uptake measurements with P-glycoprotein (*mdr1a*, *mdr1b*, *MDR1*)-expressing cell lines.

We evaluated the distribution of [^{14}C]ramosetron to the brain by using an in-situ rat and mouse brain perfusion technique. The observed PS values of [^{14}C]iodoantipyrine, [^{3}H]propranolol and [^{3}H]imipramine coincided well with the predicted values based on the octanol/water partition coefficient and molecular weight (Figure 2), suggesting that these drugs enter the brain by passive diffusion. On the other hand, the PS value of [^{14}C]ramosetron was lower than the value expected for passive diffusion (Figure 2). In other words, the brain distribution of [^{14}C]ramosetron was much lower than would be predicted from its lipophilicity, suggesting the contribution of (an) active efflux transport system(s). It is known that P-glycoprotein acts as an efflux pump at brain capillary endothelial cells to transport drugs from the brain to the circulating blood (Tatsuta et al 1992; Tsuji et al 1992; Schinkel et al 1994). Therefore, we investigated the effect of verapamil, a P-glycoprotein inhibitor, on the distribution of [^{14}C]ramosetron to the brain using an in-situ mouse brain perfusion technique. The vascular volume of [^{3}H]sucrose was not affected by verapamil or unlabelled ramosetron (Table 1). Therefore, the tight junction of brain capillary endothelial cells was not damaged by verapamil or unlabelled ramosetron. The PS value of [^{14}C]ramosetron was increased in the presence of verapamil and unlabelled ramosetron (Table 1), suggesting not that paracellular permeability was increased, but that the efflux process of [^{14}C]ramosetron was inhibited by verapamil and by a high concentration of ramosetron, and that P-glycoprotein contributes to the efflux of ramosetron at the blood–brain barrier.

Mdr1a P-glycoprotein is especially important for P-glycoprotein-mediated function at the blood–brain barrier in mice (Schinkel et al 1994). To see whether or not [^{14}C]ramosetron was transported by *mdr1a* P-glycoprotein, we examined the effect of verapamil on the uptake of [^{14}C]ramosetron by L and LV500 cells. Northern-blot analysis revealed that LV500 cells over-express *mdr1a* P-glycoprotein in comparison with the parent L cells (Kusaba et al 1995). The uptake of [^{3}H]vinblastine, which is a typical substrate of P-glycoprotein, into LV500 cells was significantly lower than that into L cells (Table 2), and the cellular accumulation into LV500 cells was increased in the presence of verapamil (Table 3). This shows that *mdr1a* P-glycoprotein expressed in LV500 cells functions as an efflux pump. The uptake of [^{14}C]ramosetron by LV500 cells was significantly lower than that by L cells (Table 2) and the steady-state uptake of [^{14}C]ramosetron by LV500 cells was increased significantly in the presence of verapamil

(Table 3). These results may reflect inhibition of the active efflux via *mdr1a* P-glycoprotein by verapamil, and suggest that [^{14}C]ramosetron was indeed extruded from the cell via *mdr1a* P-glycoprotein. These observations are in agreement with a previous report on another 5-HT_3 antagonist, ondansetron, whose transport was reported to be mediated by mouse *mdr1a* P-glycoprotein on the basis of studies using *mdr1a* (−/−) mice and LLC-PK1 cells transfected with *mdr1a* P-glycoprotein (Schinkel et al 1996).

It has been reported that *mdr1a* and *mdr1b* gene products show different transport properties for progesterone (Yang et al 1990). Previously, we observed a difference in substrate specificity between mouse *mdr1a* P-glycoprotein and *mdr1b* P-glycoprotein in a study of the efflux transport of bunitrolol, which is a hydrophobic beta blocker (Matsuzaki et al 1999). Therefore, we examined the contribution of *mdr1b* P-glycoprotein to ramosetron transport by using MBEC4 cells, which mainly express *mdr1b* P-glycoprotein (Tatsuta et al 1994). The uptake of [^{3}H]vinblastine by MBEC4 cells was significantly increased in the presence of 10, 50 and 100 μM verapamil and 20 μM ciclosporin (Table 3). On the other hand, the steady-state uptake of [^{14}C]ramosetron by MBEC4 cells was not increased by verapamil (Table 3). These results suggested that *mdr1b* P-glycoprotein makes little contribution to [^{14}C]ramosetron transport, and that there is a difference in substrate specificity between *mdr1a* P-glycoprotein and *mdr1b* P-glycoprotein. The uptake of [^{14}C]ramosetron by MBEC4 cells was significantly increased in the presence of ciclosporin (Table 3). Thus, it appears that [^{14}C]ramosetron was effluxed from the cells by a ciclosporin-sensitive transporter(s).

To define the contribution of *MDR1* P-glycoprotein to [^{14}C]ramosetron efflux, we examined the uptake of [^{14}C]ramosetron by LLC-PK1 cells and LLC-GA5-COL300 cells, which are transfected with human *MDR1* cDNA and over-express *MDR1* P-glycoprotein (Tanigawara et al 1992; Ueda et al 1992). The differences of intracellular accumulation of [^{3}H]vinblastine and inhibitory effect of ciclosporin confirmed that *MDR1* P-glycoprotein expressed in LLC-GA5-COL300 cells acts as an efflux pump for ramosetron. The uptake of [^{14}C]ramosetron by LLC-GA5-COL300 cells was significantly lower than that by LLC-PK1 cells, and was increased by verapamil and ciclosporin (Table 2 and Table 3). These results indicate that *MDR1* P-glycoprotein is involved in the efflux process of [^{14}C]ramosetron. The uptake of [^{3}H]vinblastine by LLC-PK1 cells was also increased in the presence of verapamil and ciclosporin. LLC-PK1 cells were established from porcine kidney, and P-glycoprotein is known to be expressed in kidney. Consequently, this latter effect of verapamil and ciclosporin might be accounted for by their inhibitory action on native P-glycoprotein expressed in LLC-PK1 cells.

In all the cell lines tested in this study, the uptake of [^{3}H]vinblastine was 2–3 fold greater than that of [^{14}C]ramosetron, and the uptake profiles of [^{3}H]vinblastine were different from those of [^{14}C]ramosetron. While the cell/medium ratio of [^{3}H]vinblastine slowly reached a steady state after 60 min, that of [^{14}C]ramosetron rapidly reached steady state within 15 min (data not shown). This difference may be attributed to the differences in the physicochemical properties such as lipophilicity, the contribution of influx or efflux transport system(s), changes of intracellular distribution volume owing to the binding of the drug to cytoplasmic substances or transfer into organelles, and so on. Indeed, the values of the octanol/water partition coefficient, an index of lipid solubility, of vinblastine and ramosetron were 79.6 (Murakami et al 2000) and 41.8±1.48 (mean±s.e.m.; n = 4) (this study), respectively. It is not clear why the uptake of ramosetron at 30 min was lower than that at 5 min (data not shown). One possible explanation is that an uptake transport system contributes to the influx of ramosetron, and its activity decreases in response to change in cellular ion balance with the lapse of time.

Previously, it was reported that *mdr1a* is the only isoform found in-vivo in brain capillaries in rats (Barrand et al 1995), and in mouse brain the level of *mdr1a* expression is higher than that of *mdr1b* (Jeffrey & Dieter, 1997). Our results suggest that ramosetron is transported across the blood–brain barrier by *mdr1a* P-glycoprotein and *MDR1* P-glycoprotein, but is poorly transported by *mdr1b* P-glycoprotein. Therefore, the in-vitro observations are in agreement with the in-situ data. In addition, our findings indicate that there is a difference in substrate specificity between *mdr1a* P-glycoprotein and *mdr1b* P-glycoprotein. Further studies using cells which express each P-glycoprotein individually at the same level are necessary to clarify the transport properties of *mdr1a* P-glycoprotein and *mdr1b* P-glycoprotein.

To use experimental data to predict the contribution of transporters such as P-glycoprotein to the pharmacokinetics and interactions of drugs in man, we have to take into consideration interspecies differences in the expression sites and in the level or substrate specificity of P-glycoprotein. It is known that *MDR1* P-glycoprotein is more similar to *mdr1a* P-glycoprotein than to *mdr1b* P-glycoprotein in terms of amino acid or nucleotide sequence. However, it is necessary to examine both *mdr1a* and *mdr1b* P-glycoprotein since *mdr1b* P-glycoprotein is predominantly expressed in some tissues. Although *mdr1a* P-glycoprotein plays an important role in the transport of drugs across the blood–brain barrier in mouse brain, *mdr1b* P-glycoprotein should not be disregarded because it is expressed in the brain, albeit at a low level. Many reports have described differences in the expression sites or levels of *MDR1* P-glycoprotein, *mdr1a* P-glycoprotein and *mdr1b* P-glycoprotein, but there is still little information about the substrate specificity of these P-glycoproteins. Therefore, further investigations are required to establish the differences in substrate recognition using gene over-expression systems. In this study, we examined interspecies differences of P-glycoprotein-mediated [^{14}C]ramosetron transport by using cultured cells, which over-express *MDR1* P-glycoprotein, *mdr1a* P-glycoprotein and *mdr1b* P-glycoprotein. Nevertheless, cultured cells do not exactly reflect in-vivo conditions, and there is always the possibility that cells may acquire altered protein expression during culture. As noted above, *mdr1a* P-glycoprotein is predominantly expressed in mouse brain in-vivo, and *mdr1b* P-glycoprotein

is also present at a low level. However, MBEC4 cells, which were isolated from mouse brain capillary endothelial cells and cultured, express only *mdr1b* P-glycoprotein and not *mdr1a* P-glycoprotein. These results show that an in-vitro study can not necessarily predict characteristics observed in-vivo. We therefore used both in-vitro and in-situ techniques to investigate the transport mechanism of [^{14}C] ramosetron at the blood–brain barrier, and the results demonstrated that [^{14}C]ramosetron is a substrate of *mdr1a* P-glycoprotein and *MDR1* P-glycoprotein, but not *mdr1b* P-glycoprotein. It is worth noting here that the use of knockout mice to investigate pharmacokinetics also presents problems. It was reported that *mdr1b* P-glycoprotein was induced in *mdr1a* (−/−) mice in liver and kidney but not in intestine or brain (Schinkel et al 1994). Therefore, while *mdr1a* (−/−) mice are useful to study the transport of drugs at the blood–brain barrier or secretion and absorption in the intestine, the results in liver or kidney must be interpreted with caution. Investigations from various points of view are thus necessary to clarify the contribution of carrier-mediated mechanisms to pharmacokinetics and drug interactions in man.

We can not rule out a partial contribution of other transport system(s) to the efflux of ramosetron at the blood–brain barrier, because verapamil may block such a transport system in addition to P-glycoprotein at the concentration (500 μM) we employed in this study (Table 1). An examination of the effect of verapamil on Kp of ramosetron in *mdr1a/1b*(−/−) knockout mouse using in-situ brain perfusion may provide an answer to this question. Moreover, (an)other transport system(s) may partially contribute to the efflux of ramosetron in the cultured cells studied. Indeed, the uptake of ramosetron into MBEC4 cells was increased by ciclosporin, but was not affected by 100 μM verapamil, which significantly increased the uptake of both vinblastine and ramosetron into P-glycoprotein-over-expressing cells (LLC-GA5-COL300 cells), suggesting that the efflux of ramosetron from MBEC4 cells is mediated by a transport system other than P-glycoprotein.

Conclusion

In conclusion, these results demonstrate that ramosetron is a substrate for both *mdr1a* P-glycoprotein and *MDR1* P-glycoprotein, but is poorly recognized by *mdr1b* P-glycoprotein at the blood–brain barrier. The limited distribution of ramosetron to the brain is considered to be, at least in part, due to efflux transport of ramosetron mediated by P-glycoprotein at the blood–brain barrier.

References

Barrand, M. A., Robertson, K. J., von Weikersthal, S. F. (1995) Comparison of P-glycoprotein expression in isolated rat brain microvessels and in primary cultures of endothelial cells derived from microvasculature of rat brain, epididymal fat pad and from aorta. *FEBS Lett.* **374**: 179–183

Borst, P., Schinkel, A. H., Smit, J. J., Wagenaar, E., Van Deemter, L., Smith, A. J., Eijdems, E. W., Baas, F., Zaman, G. J. (1993) Classical and novel forms of multidrug resistance and the physiological functions of P-glycoproteins in mammals. *Pharmacol. Ther.* **60**: 289–299

Cohen, D., Yang, C. P. H., Horwitz, S. (1990) The products of the *mdr1a* and *mdr1b* genes from multidrug resistant murine cells have similar degradation rates. *Life Sci.* **46**: 489–495

Cordon, C. C., O'Brien, J. P., Casals, D., Rittman-Graucer, 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. USA* **86**: 695–698

Croop, C. C., Raymond, M., Haber, D., Devault, A., Arceci, R. J., Gros, P., Housman, D. (1989) The three mouse multidrug resistance (*mdr*) genes are expressed in a tissue-specific manner in normal mouse tissues. *Mol. Cell. Biol.* **9**: 1346–1350

Devault, A., Gros, P. (1990) Two members of the mouse mdr gene family confer multidrug resistance with overlapping but distinct drug specificities. *Mol. Cell. Biol.* **10**: 1652–1663

Gros, P., Dhir, R., Croop, J., Talbot, F. (1991) A single amino acid substitution strongly modulates the activity and substrate specificity of mouse mdr1 and mdr3 drug efflux pumps. *Proc. Natl Acad. Sci. USA* **88**: 7289–7293

Hsu, S. I., Lothstein, L., Horwitz, S. B. (1989) Differential overexpression of three mdr gene family members in multidrug-resistant J774.2 mouse cells. *J. Biol. Chem.* **264**: 12053–12062

Jeffrey, A. S., Dieter, S. (1997) Expression of the multidrug resistance genes in the liver. *FASEB J.* **11**: 308–313

Juliano, R. L., Ling, V. (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochem. Biophys. Acta* **455**: 152–162

Kusaba, H., Kohno, K., Asakuno, K., Kuwano, M., Okumura, K., Green, E. D., Schlessinger, D., Wada, M. (1995) Functional expression of yeast artificial chromosome-human multidrug resistance genes in mouse cells. *Genome Res.* **5**: 245–258

Lincke, C., Smit, J., van der Velde-Koerts, T. (1991) Structure of the human *MDR 3* gene and physical mapping of the human MDR locus. *J. Biol. Chem.* **266**: 5303–5310

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275

Matsuzaki, J., Yamamoto, C., Miyama, T., Takanaga, H., Matsuo, H., Ishizuka, H., Kawahara, Y., Kuwano, M., Naito, M., Tsuruo, T., Sawada, Y. (1999) Contribution of P-glycoprotein to bunitrolol efflux across blood-brain barrier. *Biopharm. Drug Dispos.* **20**: 85–90

Murakami, H., Takanaga, H., Matsuo, H., Ohtani, H., Sawada, Y. (2000) Comparison of blood-brain barrier permeability in mice and rats using in situ brain perfusion technique. *Am. J. Physiol.* **279**: H1022–H1028

Nakamura, E., Imasaki, H., Takesige, M., Miura, H., Kobayasi, S., Higuchi, S. (1994) Metabolic fate of YM060 (1): Plasma concentration, distribution and excretion after intravenous administration of ^{14}C-YM060 to rats. *Xenobio. Metabol. Dispos.* **9**: 224–234

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) Distribution 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., 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

Takasato, Y., Rapoport, S. I., Smith, Q. R. (1984) An *in situ* brain perfusion technique to study cerebrovascular transport in the rat. *Am. J. Physiol.* **247**: H484–H493

Tanigawara, Y., Okamura, N., Hirai, M., Yasuhara, M., Ueda, K.,

Kioka, N., Komano, T., Hori, R. (1992) Transport of digitoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK1). *J. Pharmacol. Exp. Ther.* **263**: 840–845

Tatsuta, T., Naito, M., Oh-hara, T., Sugawara, I., Tsuruo, T. (1992) Functional involvement of P-glycoprotein in blood-brain barrier. *J. Biol. Chem.* **267**: 20383–20391

Tatsuta, T., Naito, M., Mikami, K., Tsuruo, T. (1994) Enhanced expression by the brain matrix of P-glycoprotein in brain capillary endothelial cells. *Cell Growth Differ.* **5**: 1145–1152

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I., Willingham, M. C. (1987) Cellular localization of the multidrug resistance gene product in normal human tissues. *Proc. Natl Acad. Sci. USA* **84**: 7735–7738

Tsuji, A., Tamai, I. (1997) Blood-brain barrier function of P-glycoprotein. *Adv. Drug Deliv. Rev.* **25**: 285–298

Tsuji, A., Terasaki, T., Takebatake, Y., Tenda, Y., Tamai, I., Yamoshima, T., Moritani, S., Tsuruo, T., Yamashita, J. (1992) P-Glycoprotein as the drug efflux pump in primary cultured bovine brain capillary endothelial cells. *Life Sci.* **51**: 1427–1437

Ueda, K., Okamura, N., Hirai, M., Tanigawara, Y., Tohru, S., Kioka, N., Komano, T., Hori, R. (1992) Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone but not progesterone. *J. Biol. Chem.* **267**: 24248–24252

Yamaoka, K., Tanigawara, Y., Nakagawa, T., Uno, T. A. (1981) Pharmacokinetic analysis program (MULTI) for the microcomputer. *J. Pharmacobiodyn.* **4**: 879–885

Yang, C. P. H., Cohen, D., Greenberger, L. M., Hsu, S., Horwitz, S. B. (1990) Differential transport properties of two *mdr* gene products are distinguished by progesterone. *J. Biol. Chem.* **265**: 10282–10288