ELSEVIER

European Journal of Pharmacology 445 (2002) 115–123

ejp

www.elsevier.com/locate/ejphar

Effect of endotoxin on doxorubicin transport across blood–brain barrier and P-glycoprotein function in mice

Ying Lan Zhao [a,b], Jun Du [a], Hiroaki Kanazawa [c], Ayako Sugawara [a], Kenji Takagi [a], Kiyoyuki Kitaichi [a], Yasuaki Tatsumi [a], Kenzo Takagi [a], Takaaki Hasegawa [a,*]

[a] *Department of Medical Technology, Nagoya University School of Health Sciences, 1-1-20 Daikominami, Higashi-ku, Nagoya 461-8673, Japan*
[b] *National Safety Assessment Center of Traditional Chinese Medicine, Sichuan University, Chengdu 610041, China*
[c] *Department of Radiological Technology, Nagoya University School of Health Sciences, 1-1-20 Daikominami, Higashi-ku, Nagoya 461-8673, Japan*

Received 22 November 2001; received in revised form 22 April 2002; accepted 23 April 2002

Abstract

The aim of this study was to investigate whether *Klebsiella pneumoniae* endotoxin modifies transport of doxorubicin, a P-glycoprotein substrate, across the blood–brain barrier and P-glycoprotein function in mice. Doxorubicin (30 mg/kg) was administered into the tail vein or fluorescein isothiocyanate-labeled dextran (FD-4) was infused (20 μg/min) into the right jugular vein of mice intravenously injected with endotoxin (10 mg/kg) 6 or 24 h earlier. Blood and brain samples were collected 4 h after injection of doxorubicin or 1 h after infusion of FD-4. We examined using Western blotting the influence of endotoxin on the expression of P-glycoprotein in brains obtained 6, 12 and 24 h after injection. Endotoxin did not change the plasma and brain concentrations and brain-to-plasma concentration ratio (K_p value) of FD-4. No histopathological changes in brain capillaries were observed. These results suggest that endotoxin does not cause damage to brain capillaries. Plasma and brain concentrations of doxorubicin in mice treated 6 h earlier with endotoxin were significantly higher than those in control and mice treated 24 h earlier. However, endotoxin did not significantly change the K_p value of doxorubicin. The protein level of P-glycoprotein was significantly, but slightly down-regulated 6 h after endotoxin treatment. However, the levels remained almost unchanged after 12 and 24 h. The present results suggest that *Klebsiella pneumoniae* endotoxin has no effect on the brain capillary integrity and doxorubicin transport across the blood–brain barrier in mice. It is likely that P-glycoprotein function might be sufficient to transport doxorubicin in spite of decreased levels of P-glycoprotein in the brain.

Keywords: Endotoxin; P-Glycoprotein; Blood–brain barrier; Doxorubicin

1. Introduction

It is well known that numerous cancer cells overexpress multidrug resistance (MDR) gene-encoded P-glycoprotein, resulting in anticancer drug resistance (Juliano and Ling, 1976; Ueda et al., 1987). P-glycoprotein, a member of the ATP-binding cassette superfamily of transport proteins, acts as an active efflux pump for hydrophobic and cationic anticancer drugs, such as *Vinca* alkaloids and anthracycline antibiotics, and plays an important role in their antitumor activity (Endicott and Ling, 1989; Tsuruo, 1988; Tsuruo et al., 1982).

P-Glycoprotein is expressed largely, not only in anticancer drug resistant cells, but also in various normal tissues such as liver, kidneys, intestine and brain (Cordon-Cardo et al., 1990; Thiebaut et al., 1987). In these tissues, this protein has a protective function of excluding endogenous and exogenous substances from the body. In brain, P-glycoprotein is expressed in the luminal membrane of the brain capillary endothelium (Cordon-Cardo et al., 1989; Schinkel et al., 1994) and in the apical membrane of the choroid plexus epithelium (Rao, 1999). In particular, P-glycoprotein in the blood–brain barrier has an important role in limiting the distribution of various substances into the brain to protect from neurotoxicity (Ohnishi et al., 1995; Tsuji, 1998; Watchko et al., 1998). It is likely that the existence of P-glycoprotein in the blood–brain barrier also leads to chemotherapy failure in brain cancers by limiting the effectiveness of many anticancer drugs since MDR 1 is expressed in human brain tumors (Nabors et al., 1991). Ohnishi et al. (1995) have reported that the brain distribution of doxorubicin, a substrate of P-glycoprotein, is mainly

* Corresponding author. Tel.: +81-52-719-3008; fax: +81-52-719-3009.
E-mail address: hasegawa@met.nagoya-u.ac.jp (T. Hasegawa).

0014-2999/02/$ - see front matter
PII: S0014-2999(02)01661-8

restricted by P-glycoprotein on the blood–brain barrier under normal physiological conditions. It is thought that the integrity of brain capillary cells and the function of P-glycoprotein in the blood–brain barrier may be modified by histopathological and physiological changes occurring in various disease states, including bacterial infectious disease.

Endotoxin, an active component in the outer membrane of Gram-negative bacteria, is known for its various biological and immunological activities. Endotoxin has been found to induce various histopathological and physiological changes in the body, such as damage to the central nervous system, liver, kidney, heart, gastrointestinal tract and lungs (Hewett and Roth, 1993). We have reported that *K. pneumoniae* endotoxin reduces hepatic drug-metabolizing enzyme activity and impairs biliary and renal excretion of various drugs by changing the ability of the biliary and tubular secretory systems (Haghgoo et al., 1995; Hasegawa et al., 1994; Kitaichi et al., 1999; Nadai et al., 1993a,b,c, 1996, 1998). In addition, we recently reported that *K. pneumoniae* endotoxin impaired the P-glycoprotein-mediated transport of P-glycoprotein substrates, rhodamine 123 and sparfloxacin in rats (Ando et al., 2001; Nadai et al., 2001).

There are many reports regarding the effects of endotoxin and endotoxin-induced inflammatory cytokines and mediators on brain permeability (Boje, 1996; De Vries et al., 1996; Mayhan, 1998; Shukla et al., 1995). However, almost all the studies were carried out in vitro with brain endothelial cells and the in vivo studies used topical administration of endotoxin in animals. There is little in vivo information available regarding the effect of systemic administration of endotoxin on blood–brain barrier integrity and P-glycoprotein function.

The purpose of the present study was to investigate whether *K. pneumoniae* endotoxin changes the in vivo blood–brain barrier transport of doxorubicin and P-glycoprotein function. We measured the plasma and brain concentrations and brain-to-plasma concentration ratio of doxorubicin or of fluorescein isothiocyanate-labeled dextran and histopathological changes in brain capillaries. Furthermore, the effect of endotoxin on the constitutive level of P-glycoprotein in the brain was examined using Western blot analysis.

2. Methods

2.1. Materials

Endotoxin was isolated from *K. pneumoniae* LEN-1 (O3: K1$^-$), which was identical to that used in previous studies (Ando et al., 2001; Kitaichi et al., 1999; Nadai et al., 1998). Doxorubicin hydrochloride, daunorubicin hydrochloride and fluorescein isothiocyanate-labeled dextran with average molecular weight 4.4 kDa (FD-4) were purchased from Sigma (St. Louis, MO). Doxorubicin hydrochloride in the form of a commercial preparation for injection (Adriamycin) was obtained from Kyowa Hakko (Tokyo, Japan). Cyclosporin A was purchased from Novartis Pharma (Tokyo, Japan). All other reagents were commercially available and were of analytical grade. All chemicals were dissolved in physiological saline before use.

2.2. Animals

Male ddY mice (Japan SLC, Hamamatsu, Japan), weighing 25–28 g, were used in this study. The mice were housed under controlled environmental conditions (approximately 25 °C) with a commercial food and water freely available. All animal experiments were carried out in accordance with the guidelines of Nagoya University School of Medicine for the care and use of laboratory animals.

2.3. Experimental protocols

For the blood–brain distribution experiments, *K. pneumoniae* endotoxin (10 mg/kg) was administered into the tail vein of ddY mice 6 or 24 h before injection of each drug. Control mice were treated with isotonic saline in place of endotoxin. The dose of endotoxin was based on that in our previous study (Nadai et al., 1996) and on data for the LD_{50} (15 mg/kg) to mice.

To elucidate the time-dependent effect of endotoxin on blood–brain barrier integrity, the mice were anesthetized with pentobarbital (25 mg/kg) and were cannulated with polyethylene tubes (PE 10, Natsume, Tokyo, Japan), in the right jugular vein for drug administration. After the completion of surgical preparation, the mice received a constant rate infusion of FD-4 (20 μg/min) at a rate of 5 μl/min by means of a Harvard infusion pump (PHD 2000, South Natick, MA). A 60-min infusion was found to result in a steady state concentration of FD-4. The dosage of FD-4 was calculated from the pharmacokinetic parameters as described previously (Mehvar and Shepard, 1992). After 60 min of FD-4 infusion, the animals were killed by exsanguination, and brain and blood samples were taken. This experiment was done under pentobarbital anesthesia and the body temperature was maintained at 37 °C with a heat lamp.

To examine the time-dependent effect of endotoxin on doxorubicin brain distribution, doxorubicin (30 mg/kg) was administered into the tail vein of mice without anesthesia. The dosage was chosen so as to allow us to measure reproducibly the concentration of doxorubicin in the brain, as concentration in the brain was not detectable, using the method described above, 4 h after administration of doxorubicin at a dose of 10 mg/kg. The mice were killed by exsanguination under light ether anesthesia 4 h after injection of doxorubicin, and blood and brain were removed.

To examine the effect of a typical P-glycoprotein inhibitor, cyclosporin A, on the brain distribution of doxorubicin, cyclosporin A (200 mg/kg) was administered intraperitoneally to mice 30 min before injection of doxorubicin (30

mg/kg). The mice were killed by exsanguination under light ether anesthesia 4 h after injection of doxorubicin, and blood and brain were removed.

Blood samples were immediately centrifuged to yield plasma. The brain was weighed. All plasma and brain samples were stored at −80 °C until assay.

The K_p value of doxorubicin or FD-4 is presented as the brain-to-plasma concentration ratio.

2.4. Concentrations of nitrite and nitrate (NO_x) in serum and brain

To measure the concentration of nitrite and nitrate (NO_x) in serum and brain, blood and brain samples were taken 12 h after injection of endotoxin (10 mg/kg), based on our previous report (Kitaichi et al., 1999). Concentrations of NO_x were measured with a commercial kit (Nitrate/Nitrite Colorimetric Assay Kit: Cayman Chemical, Ann Arbor, MI) according to the manufacturers' instructions. The detection limit for NO_x by this method was determined to be 2.5 μM, and values below 2.5 μM are given as a not-detected (N.D.) value.

2.5. Histopathological examination

Mice were killed by exsanguination under light ether anesthesia 6, 12 and 24 h after an intravenous injection of endotoxin (10 mg/kg) and the brain was removed immediately. Control mice were treated with isotonic saline in place of endotoxin. For electron microscopy, small pieces of brain tissue were fixed in phosphate-buffered 2.5% glutaraldehyde (0.1 M phosphate buffer, pH 7.4) at 4 °C. After being rinsed in the buffer, the specimens were postfixed in 1% osmium tetroxide, and embedded in Epon 812 resin. Ultrathin sections were cut on a Reichert Ultracut-N ultramicrotome with a diamond knife, double stained with uranyl acetate and lead citrate, and examined in a Hitachi H-7000 electron microscope. Veterinary pathologists performed the histopathological examinations.

2.6. Western blot analysis

Brains were taken from mice treated 6, 12 and 24 h earlier with endotoxin. Each brain was suspended in 1 ml of 10 mM Tris–HCl buffer (pH 8.0) containing complete protease inhibitor, 1.5 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (Sigma Chemicals). The suspension was homogenized with a tight homogenizer (twenty strokes up and down) and centrifuged for 10 min at 3500 × *g* at 4 °C. The supernatant was centrifuged for 60 min at 80,000*g* at 4 °C. The pellet was dissolved in Laemmli buffer and incubated for 15 min at 37 °C.

The protein concentration in the solution was measured with a Bio-Rad Protein Assay (Bio-Rad Laboratories, CA, USA) using bovine serum albumin (Sigma) as a standard. The protein (40 μg) was separated by electrophoresis on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 5% nonfat dry milk, detected by C219 mouse monoclonal antibody (DAKO, Glostrup, Denmark). To quantify the relative levels of P-glycoprotein in each gel, the intensity of the stained bands was measured by NIH image (Bethesda, MD).

2.7. Drug assay

Plasma samples were diluted appropriately with distilled water and brains were homogenized in a fourfold volume of PBS solution. The diluted plasma samples and the homogenates were used immediately for measurement of FD-4. The concentration of FD-4 in each sample was measured with a spectrofluorophotometer (RF-1500, Shimadzu, Kyoto, Japan) set at an excitation wavelength of 498 nm and an emission wavelength of 525 nm.

The concentration of doxorubicin in plasma and brain was determined by high-performance liquid chromatography (HPLC). Briefly, 50 μl of each sample was vortexed with 300 μl of acetonitrile containing an internal standard of daunorubicin (0.6 μg/ml). After centrifugation, 300 μl of the supernatant was evaporated to dryness under a nitrogen gas stream at 45 °C. The residue was reconstituted with 200 μl of the mobile phase and analyzed by HPLC. The apparatus used for HPLC was a Shimadzu LC-6A system (Kyoto, Japan) consisting of an LC-6A liquid pump and an SIL-6A autoinjector, and was equipped with a fluorescence detector (RF-535, Shimadzu) set at an excitation wavelength of 475 nm and emission wavelength of 580 nm. Conditions were as follows: column, a Cosmocil $5C_{18}$ column (Nacalai Tesque, Kyoto, Japan); mobile phase, 10 mM citric acid/NH_4HPO_4 buffer (pH 4.0)–acetonitrile = 2:1 (v/v); temperature, 50 °C; flow rate, 1 ml/min. The detection limit of this assay was 40 ng/ml for plasma and 50 ng/g for brain.

2.8. Statistical analysis

All data are expressed as means ± S.E.M. Analysis of variance (ANOVA) was used to determine the statistical significance of differences between experimental groups. When F ratios were significant, Scheffe's post hoc tests between two groups were done. The 0.05 level of probability was used as criterion of significance.

3. Results

3.1. Effect of endotoxin on the brain distribution of FD-4

Fig. 1 shows the brain to plasma concentration ratio (K_p) of FD-4 60 min after the start of FD-4 infusion to mice pretreated or not with endotoxin. The K_p value of FD-4 in the control mice was 0.07 ± 0.01 ml/g brain, which was not

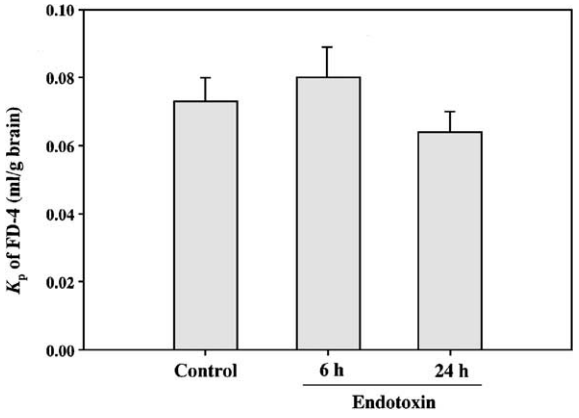

Fig. 1. Effect of endotoxin on brain to plasma concentration ratio (K_p) of FD-4. Brain and plasma samples were taken 60 min after infusion of FD-4. Each column represents mean ± S.E.M. ($n = 6–9$). No significant differences were observed between the control mice and mice treated 6 or 24 h earlier with endotoxin.

significantly different from those for mice treated 6 and 24 h earlier with endotoxin (0.08 ± 0.01 and 0.06 ± 0.01 ml/g brain, respectively). No significant differences in the plasma and brain concentrations of FD-4 were observed between the three groups. These results indicate the maintenance of blood–brain barrier integrity in endotoxin-treated mice.

3.2. Effect of endotoxin on the brain distribution of doxorubicin

Table 1 shows the plasma and brain concentrations and the K_p value of doxorubicin at 4 h after intravenous injection to mice pretreated or not with endotoxin. There were no significant differences in the K_p value of doxorubicin between the control mice (0.38 ± 0.02 ml/g brain) and mice treated 6 or 24 h earlier with endotoxin (0.42 ± 0.03 or 0.36 ± 0.01 ml/g brain). On the other hand, the concentrations of doxorubicin in the plasma and brain were significantly higher in mice treated 6 h earlier after injection

Table 1
Effect of endotoxin on doxorubicin transport across blood–brain barrier in mice

	Control	Time after injection of endotoxin (h)	
		6	24
Plasma level (μg/ml)	0.268 ± 0.013	0.324 ± 0.027[a]	0.249 ± 0.004
Brain level (μg/g brain)	0.101 ± 0.002	0.133 ± 0.010[a]	0.090 ± 0.004
K_p value (ml/g brain)	0.384 ± 0.023	0.415 ± 0.030	0.361 ± 0.013

Doxorubicin (30 mg/kg) was administered into the tail vein of mice 6 and 24 h after intravenous injection of endotoxin (10 mg/kg) or saline. K_p value was calculated as the ratio of brain tissue doxorubicin concentration to plasma level. Blood and brain samples were collected 4 h after injection of doxorubicin. Each value is presented as mean ± S.E.M. ($n = 6$).

[a] Significantly different from the control ($P < 0.05$).

Table 2
Effect of cyclosporin A on doxorubicin transport across blood–brain barrier in mice

	Control	Cyclosporin A
Plasma level (μg/ml)	0.263 ± 0.014	0.444 ± 0.036[a]
Brain level (μg/g brain)	0.120 ± 0.016	0.362 ± 0.054[a]
K_p value (ml/g brain)	0.457 ± 0.046	0.808 ± 0.066[a]

Doxorubicin (30 mg/kg) was administered into the tail vein of mice 30 min after intraperitoneal injection of cyclosporin A (200 mg/kg) or saline. Blood and brain samples were collected 4 h after injection of doxorubicin. Each value is presented as mean ± S.E.M. ($n = 7$).

[a] Significantly different from the control ($P < 0.05$).

of endotoxin than those in the control mice and mice treated 24 h earlier. The finding of no change in the K_p value of doxorubicin suggests that endotoxin has no effect on the blood–brain barrier transport of doxorubicin.

3.3. Concentrations of NO_x in serum and brain

We investigated the effect of endotoxin on the level of NO_x in the serum and brain. Endotoxin significantly increased the level of NO_x in the serum compared to the control mice (15.6 ± 3.6 to 651.9 ± 66.7 μM). However, NO_x was not detected in the brain of either the control or endotoxin-treated mice.

3.4. Effect of cyclosporin A on the brain distribution of doxorubicin

Table 2 shows the effect of cyclosporin A (200 mg/kg) on the plasma and brain concentrations and the K_p value of doxorubicin. Pretreatment with cyclosporin A increased the plasma concentration of doxorubicin 1.7-fold (0.26–0.44

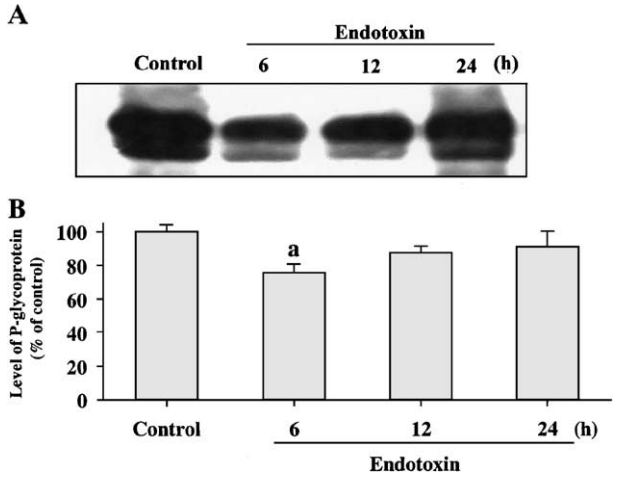

Fig. 2. P-Glycoprotein expression in brain of mice treated or not with endotoxin (A) and ratio of relative staining intensity for P-glycoprotein in control and endotoxin-treated mice (B). Brain samples were taken 6, 12 and 24 h after intravenous injection of endotoxin (10 mg/kg). The band corresponded to 160–170 kDa. Each column represents the intensity ratio for the control mice and is presented as mean ± S.E.M. ($n = 5$). [a]Significantly different from the control ($P < 0.05$).

μg/ml) and the brain concentration threefold (0.12–0.36 μg/g brain). The K_p value in mice pretreated with cyclosporin A was significantly higher than that in control mice (0.81 ± 0.07 and 0.46 ± 0.05 ml/g brain, respectively). These results indicate that cyclosporin A inhibits the P-glycoprotein efflux pump in the brain.

3.5. Western blot analysis of P-glycoprotein in the brain

Fig. 2A shows Western blots of P-glycoprotein expression after injection of endotoxin. Western blots revealed a band of 160–170 kDa, corresponding to P-glycoprotein. As illustrated in Fig. 2B, the protein level of P-glycoprotein was significantly down-regulated to 76% of the control after 6 h of endotoxin treatment. The level of P-glycoprotein expression at 12 and 24 h, however, was not significant. These results suggest that endotoxin down-regulates the expression of P-glycoprotein in the early phase after treatment.

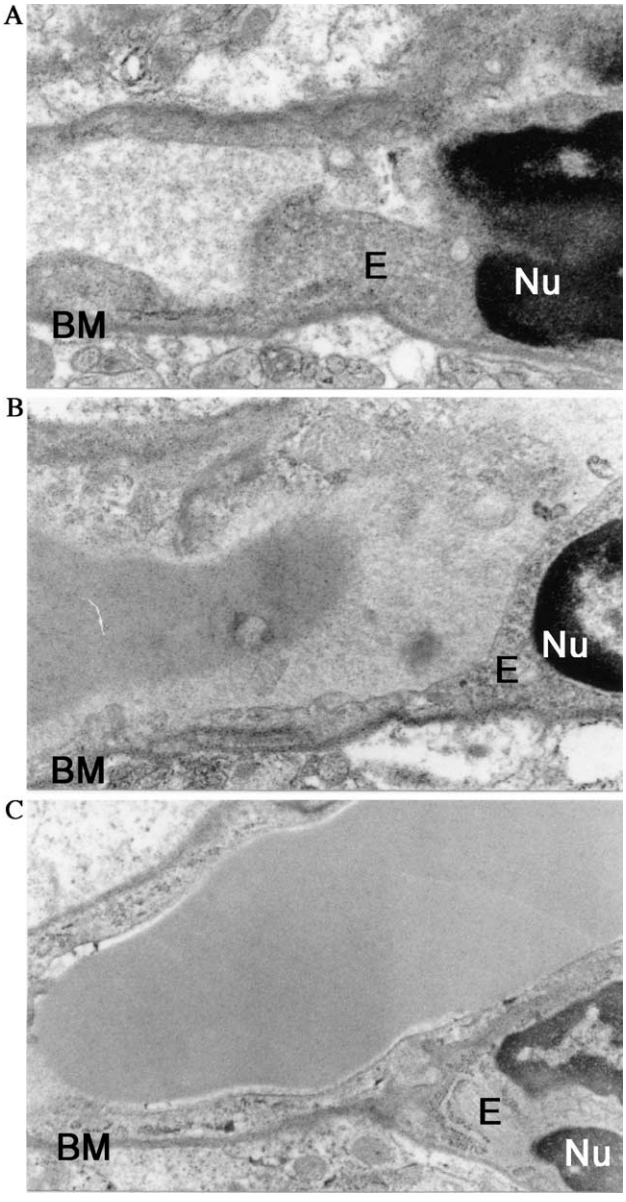

Fig. 3. Electron microscopy of brain capillaries of mice treated or not with endotoxin. (A) Control (saline). (B) Six hours after an intravenous injection of endotoxin (10 mg/kg). (C) Twenty-four hours after an intravenous injection of endotoxin (10 mg/kg). There were no marked changes in the nucleus (Nu) and cytoplasm (E) of the capillary endothelial cells. BM, basement membranes. a, b, c: × 22,000.

3.6. Histopathological examination of brain

The dose of endotoxin used in this study did not induce any histopathological changes in the brain capillaries and their basement membranes (Fig. 3). The histopathological experiments suggest the absence of any brain capillary damage caused by endotoxin treatment.

4. Discussion

Our interest in the effect of endotoxin on the brain distribution of drugs was stimulated by the reports that endotoxin at a high dose does not damage the blood–brain barrier in rats (Bickel et al., 1998). We have extensively studied the effect of *K. pneumoniae* endotoxin on the biliary and renal excretion of various drugs and hepatic drug-metabolizing enzymes in mice and rats (Ando et al., 2001; Haghgoo et al., 1995; Hasegawa et al., 1994; Kitaichi et al., 1999; Nadai et al., 1993a,b,c, 1996, 1998, in press; Wang et al., 1993). We designed the present study to investigate the effect of *K. pneumoniae* endotoxin on the brain distribution of doxorubicin, a P-glycoprotein substrate, in mice and on the expression of P-glycoprotein in the brain.

First, we measured the K_p value of FD-4, an integrity marker (Van Bree et al., 1988), to evaluate whether endotoxin could alter blood–brain barrier integrity. Histopathological examination was also performed to evaluate possible alterations of brain capillary cell function. Endotoxin did not change the K_p value of FD-4 or induce histopathological changes in the brain capillaries, indicating maintenance of the blood–brain barrier integrity. The present finding of no change in the K_p value of FD-4 in endotoxin-treated mice is supported by previous observations in rats, using sucrose and fluorescein which have a much smaller molecular weight than FD-4 (Bickel et al., 1998; De Vries et al., 1995). On the contrary, it is reported that *E. coli* endotoxin increased the permeability of fluorescein in in vitro experiments using endothelial cells from bovine brain (De Vries et al., 1996). The reason for the discrepancy between these results and ours is not clear at present, although it might be a result of the different methods, different species and the use of different endotoxin. We examined the effect of endotoxin on the transport of doxorubicin across the blood–brain barrier and found that endotoxin did not change the K_p value of doxorubicin. This indicated that doxorubicin transport across the blood–brain barrier remained unchanged under our

conditions. Furthermore, we examined the time-dependent effect of endotoxin on the expression of P-glycoprotein in the brain by Western blot analysis. Unexpectedly, a significant decrease in the protein level of P-glycoprotein was observed in the brain of mice treated 6 h earlier with endotoxin, but the reduction was slight. Based on these results, we believe that it is likely that P-glycoprotein preserves sufficient function as an efflux pump for its substrates under our conditions. However, since the brain was not perfused prior to dissection, blood vessels may still have been filled with blood and hence lymphocytes expressing P-glycoprotein. There is a possibility that endotoxin decreases the expression of P-glycoprotein in these cells rather than in the brain. On the other hand, the present study showed significant increases in the plasma and brain concentrations of doxorubicin in mice treated 6 h earlier with endotoxin, although endotoxin did not alter the K_p value. The predominant pathway for the elimination of doxorubicin is biliary and renal excretion (Kiso et al., 2000; Speeg and Maldonado, 1994; Tavoloni and Guarino, 1980; Van Asperen et al., 2000; Zhao et al., 2002). We recently demonstrated that endotoxin decreased the renal and biliary excretion of rhodamine 123, a typical P-glycoprotein substrate, and the expression of mdr1a mRNA in the liver and kidney 6 h after injection (Ando et al., 2001). It seems that the increased plasma and brain concentrations of doxorubicin may have been due to changes occurring in the peripheral tissues, but not in the brain.

Doxorubicin is known to be metabolized by aldo-ketoreductase, Cytochrome *P*450 (including CYP2B1) and glucuronide-conjugating enzymes (Arcamone et al., 1984; Ballet et al., 1986; Goeptar et al., 1993; Van Asperen et al., 2000; Weenen et al., 1984). We have recently found that *K. pneumoniae* endotoxin has no effect on glucuronidation activity in vitro (Nadai et al., in press), suggesting that endotoxin does not affect glucuronoconjugation. On the other hand, endotoxin reduces the total hepatic Cytochrome *P*450 content, impairs P450 catalytic activity and down-regulates the expression of several P450 isozymes 2C11, 2E1 and 3A2 (Gorodischer et al., 1976; Kitaichi et al., 1999; Morgan, 1989, 1997; Sewer et al., 1996). Most recently, Ferrari et al. (2001) reported that endotoxin down-regulated CYP2B1 protein in rat hepatocytes, suggesting the possibility that the plasma concentration of doxorubicin increases as a result of deterioration of CYP2B1-mediated hepatic metabolism of doxorubicin. On the other hand, some reports showed that endotoxin administered centrally induces down-regulation of Cytochrome *P*450 in the brain (Renton et al., 1999; Renton and Nicholson, 2000). Interestingly, Renton and Nicholson (2000) demonstrated that intraperitoneal injection of high doses of endotoxin decreases Cytochrome *P*450 activity in rat brain and liver. It has been suggested that, among the major metabolites of doxorubicin, 7-deoxydoxorubicinolone and doxorubicinol are substrates for P-glycoprotein (Van Asperen et al., 2000). However, the metabolism of doxorubicin in the brain remains to be elucidated. Based on these findings and on the present data, it is possible that the endotoxin-induced increase in the plasma and brain concentrations of doxorubicin may be in part caused by reduction in hepatic deletes drug-metabolizing enzyme activity.

It is known that protein binding plays an important role in the distribution of drugs, since only the unbound drug portion is distributed into target tissues, such as brain. The K_p value of doxorubicin might also be affected by changes in the protein binding. Our previous study showed that the protein binding potency of doxorubicin in rats was relatively low (approximately 60%) (Kiso et al., 2000). It is likely that endotoxin does not alter the unbound fraction of doxorubicin in plasma. There are several reports showing that endotoxin can cause damage to numerous organs and changes the expression of various drug transporters in the liver (Hartmann et al., 2001; Hewett and Roth, 1993; Tang et al., 2000; Vos et al., 1998). Since the brain is a privileged organ due to the blood–brain barrier, it seems likely that the effect of endotoxin on the brain is distinct from that on other peripheral tissues such as kidneys and liver.

Under our conditions, cyclosporin A, an immunosuppressive agent that has been well established as an inhibitor of P-glycoprotein, significantly increased the plasma and brain concentrations and K_p value of doxorubicin. Results obtained from the present study are supported by reports showing that cyclosporin A increases the brain tissue concentration of doxorubicin (Hughes et al., 1998; Saito et al., 2001; Zhang et al., 2000). On the contrary, there are several reports that cyclosporin A and verapamil did not modify the brain permeability of doxorubicin (Colombo et al., 1994; Rousselle et al., 2000; Warren et al., 2000). The reasons for the discrepancies among these reports are not clear at present, although they might be a result of the different methods used. One possibility is that the concentration of cyclosporin A in the brain might be not sufficient to inhibit P-glycoprotein. It is also reported that doxorubicin in combination with a P-glycoprotein inhibitor (S9788) induces disruption of the blood–brain barrier in vitro (Fenart et al., 1998). Therefore, the observed increase in the brain penetration of doxorubicin may also be partially explained by blood–brain barrier disruption after coadministration with cyclosporin A. The cyclosporin A-induced increase in the plasma and brain concentrations of doxorubicin might also be caused by reduction of P-glycoprotein-mediated biliary and renal excretion of doxorubicin since it is known that P-glycoprotein plays a role in the biliary and urinary excretion of doxorubicin (Kiso et al., 2000; Saito et al., 2001; Speeg and Maldonado, 1994; Van Asperen et al., 2000). It is likely that cyclosporin A modifies doxorubicin transport across the blood–brain barrier by inhibiting P-glycoprotein.

It is well known that various proinflammatory cytokines and mediators released by endotoxin, such as tumor necrosis factor-α (TNF-α), interleukin-1, interleukin-6, nitric oxide (NO) and prostaglandins, play important roles in endotoxin-induced pathophysiological changes in peripheral circula-

tion and tissues. It has been suggested that central injection of endotoxin induces inducible nitric oxide synthase (iNOS) activity and produces NO in the brain and leads to an increase in the permeability of drug with a small molecular weight into the brain due to damage to the blood–brain barrier (Boje, 1996; Shukla et al., 1995). On the other hand, endotoxin has been shown to induce iNOS in rat brain by intravenous injection of 5 mg/kg (Lomniczi et al., 2000) and by peritoneal injection of 25 mg/kg (Chikada et al., 2000). TNF-α was also shown to damage the blood–brain barrier (Liu et al., 1996). It appears that NO and the other proinflammatory cytokines might be important in modifying the permeability of the blood–brain barrier. Considering that TNF-α and NO can easily penetrate the barrier when damage of the blood–brain barrier occurs, we, therefore, measured the concentrations of NO_x in the serum and brain. Endotoxin dramatically increased NO_x levels in the serum as reported previously (Kitaichi et al., 1999). NO_x, however, were not detected in the brain. At present, the discrepancy between the present results and those of studies by Chikada et al. (2000) and Lomniczi et al. (2000) cannot be explained clearly. Considering that different bacterial strains were shown to affect bacterial activity and cytokine induction (Flad et al., 1993), the discrepancy may be due to a bacterial strain difference between *E. coli* and *K. pneumonia*e and/or a species difference between rats and mice. It is most probable that circulating cytokines do not enter the brain through the blood–brain barrier unless the blood–brain barrier is injured and that NO may rapidly disappear from the systemic circulation after its production.

In conclusion, the present study demonstrated that *K. pneumoniae* endotoxin does not impair blood–brain barrier integrity and doxorubicin transport across the blood–brain barrier in mice. This study is the first to describe the effect of endotoxin on the protein level of P-glycoprotein in the brain. More studies are needed to elucidate the mechanism by which endotoxin down-regulates the expression of P-glycoprotein in the brain.

Acknowledgements

This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, Sport and Culture of Japan and by a grant from the Imanaga Medical Foundation.

References

Ando, H., Nishio, Y., Ito, K., Nakao, A., Wang, L., Zhao, Y.L., Kitaichi, K., Takagi, K., Hasegawa, T., 2001. Effect of endotoxin on P-glycoprotein-mediated biliary and renal excretion of rhodamine-123 in rats. Antimicrob. Agents Chemother. 45, 3462–3467.

Arcamone, F., Lazzati, M., Vicario, G.P., Zini, G., 1984. Disposition of ^{14}C-labelled 4′-epidoxorubicin and doxorubicin in the rat. A comparative study. Cancer Chemother. Pharmacol. 12, 157–166.

Ballet, F., Robert, J., Bouma, M.E., Vrignaud, P., Infante, R., 1986. Metabolism of doxorubicin and epirubicin in adult human hepatocytes in culture. Pharmacol. Res. Commun. 18, 343–347.

Bickel, U., Grave, B., Kang, Y.S., del Rey, A., Voigt, K., 1998. No increase in blood–brain barrier permeability after intraperitoneal injection of endotoxin in the rat. J. Neuroimmunol. 85, 131–136.

Boje, K.M., 1996. Inhibition of nitric oxide synthase attenuates blood–brain barrier disruption during experimental meningitis. Brain Res. 720, 75–83.

Chikada, N., Imaki, T., Harada, S., Nakajima, K., Naruse, M., Yoshimoto, T., Seki, T., Tanabe, A., Takano, K., 2000. A defect of lipopolysaccharide-induced nitric oxide synthase gene expression in the paraventricular nucleus of Lewis rats. Endocr. J. 47, 221–229.

Colombo, T., Zucchetti, M., D'Incalci, M., 1994. Cyclosporin A markedly changes the distribution of doxorubicin in mice and rats. J. Pharmacol. Exp. Ther. 269, 22–27.

Cordon-Cardo, B., 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, 689–695.

Cordon-Cardo, B., O'Brien, J.P., Casals, D., Bertino, J.R., Melamed, M.R., 1990. Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. J. Histochem. Cytochem. 38, 1272–1275.

De Vries, H.E., Eppens, E.F., Prins, M., Kuiper, J., Van Berkel, T.J., De Boer, A.G., Breimer, D.D., 1995. Transport of a hydrophilic compound into the cerebrospinal fluid during experimental allergic encephalomyelitis and after lipopolysaccharide administration. Pharm. Res. 12, 1932–1936.

De Vries, H.E., Blom-Roosemalen, M.C.M., De Boer, A.G., Van Berkel, T.J.C., Breimer, D.D., Kuiper, J., 1996. Effect of endotoxin on permeability of bovine cerebral endothelial cell layers in vitro. J. Pharmacol. Exp. Ther. 277, 1418–1423.

Endicott, J.A., Ling, V., 1989. The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu. Rev. Biochem. 58, 137–171.

Fenart, L., Buee-Scherrer, V., Descamps, L., Duhem, C., Poullain, M.G., Cecchelli, R., Dehouck, M.P., 1998. Inhibition of P-glycoprotein: rapid assessment of its implication in blood–brain barrier integrity and drug transport to the brain by an in vitro model of the blood–brain barrier. Pharm. Res. 15, 993–1000.

Ferrari, L., Peng, N., Halpert, J.R., Morgan, E.T., 2001. Role of nitric oxide in down-regulation of CYP2B1 protein, but not RNA, in primary cultures of rat hepatocytes. Mol. Pharmacol. 60, 209–216.

Flad, H.D., Loppnow, H., Rietschel, E.T., Uimer, A.J., 1993. Agonists and antagonists for lipopolysaccharide-induced cytokines. Immunobiology 187, 303–316.

Goeptar, A.R., Te Koppele, J.M., Lamime, E.K., Pique, J.M., Vermeulen, N.P., 1993. Cytochrome *P*450 2B1-mediated one-electron reduction of adriamycin: a study with rat liver microsomes and purified enzymes. Mol. Pharmacol. 44, 1267–1277.

Gorodischer, R., Larsner, J., McDevitt, J.J., Nolan, J.P., Yaffe, S.J., 1976. Hepatic microsomal drug metabolism after administration of endotoxin in rats. Biochem. Pharmacol. 25, 351–353.

Haghgoo, S., Hasegawa, T., Nadai, M., Wang, L., Nabeshima, T., Kato, N., 1995. Effect of a bacterial lipopolysaccharide on biliary excretion of a β-lactam antibiotic, cefoperazone, in rats. Antimicrob. Agents Chemother. 39, 2258–2261.

Hartmann, G., Kim, H., Piquette-Miller, M., 2001. Regulation of the hepatic multidrug resistance gene expression by endotoxin and inflammatory cytokines in mice. Int. Immunopharmacol. 1, 189–199.

Hasegawa, T., Nadai, M., Wang, L., Takayama, Y., Kato, K., Nabeshima, T., Kato, N., 1994. Renal excretion of famotidine and role of adenosine in renal failure induced by bacterial lipopolysaccharide in rats. Drug Metab. Dispos. 22, 8–13.

Hewett, J.A., Roth, R.A., 1993. Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. Pharmacol. Rev. 45, 381–411.

Hughes, C.S., Vaden, S.L., Manaugh, C.A., Price, G.S., Hudson, L.C.,

1998. Modulation of doxorubicin concentration by cyclosporin A in brain and testicular barrier tissues expressing P-glycoprotein in rats. J. Neuro-Oncol. 37, 45–54.

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

Kiso, S., Cai, S.H., Kitaichi, K., Furui, N., Takagi, K., Takagi, K., Nabeshima, T., Hasegawa, T., 2000. Inhibitory effect of erythromycin on P-glycoprotein-mediated biliary excretion of doxorubicin in rats. Anticancer Res. 20, 2827–2834.

Kitaichi, K., Wang, L., Takagi, K., Iwase, M., Shibata, E., Nadai, M., Takagi, K., Hasegawa, T., 1999. Decreased antipyrine clearance following endotoxin administration: in vivo evidence of the role of nitric oxide. Antimicrob. Agents Chemother. 43, 2697–2701.

Liu, L., Kita, T., Tanaka, N., Kinoshita, Y., 1996. The expression of tumor necrosis factor in the hypothalamus after treatment with lipopolysaccharide. Int. J. Exp. Pathol. 77, 37–44.

Lomniczi, A., Cebral, E., Canteros, G., McCann, S.M., Rettori, V., 2000. Methylene blue inhibits the increase of inducible nitric oxide synthase activity induced by stress and lipopolysaccharide in the medial basal hypothalamus of rats. Neuroimmunomodulation 8, 122–127.

Mayhan, W.G., 1998. Effect of lipopolysaccharide on the permeability and reactivity of the cerebral microcirculation: role of inducible nitric oxide synthase. Brain Res. 792, 353–357.

Mehvar, R., Shepard, T.L., 1992. Molecular-weight-dependent pharmacokinetics of fluorescein-labeled dextrans in rats. J. Pharm. Sci. 81, 908–912.

Morgan, E.T., 1989. Suppression of constitutive Cytochrome *P*-450 gene expression in livers of rats undergoing an acute phase response to endotoxin. Mol. Pharmacol. 36, 699–707.

Morgan, E.T., 1997. Regulation of Cytochrome *P*450 during inflammation and infection. Drug Metab. Rev. 29, 1129–1188.

Nabors, M.W., Griffin, M.D., Zehnbauer, B.A., Hruban, R.H., Philips, P.C., Grossman, S.A., Brem, H., Colvin, O.M., 1991. Multidrug resistance gene (MDR1) expression in human brain tumors. J. Neurosurg. 75, 941–946.

Nadai, M., Hasegawa, T., Kato, K., Wang, L., Nabeshima, T., Kato, N., 1993a. Influence of a bacterial lipopolysaccharide on the pharmacokinetics of tobramycin in rats. J. Pharm. Pharmacol. 45, 971–974.

Nadai, M., Hasegawa, T., Kato, K., Wang, L., Nabeshima, T., Kato, N., 1993b. Alterations in pharmacokinetics and protein binding behavior of cefazolin in endotoxemic rats. Antimicrob. Agents Chemother. 37, 1781–1785.

Nadai, M., Hasegawa, T., Kato, K., Wang, L., Nabeshima, T., Kato, N., 1993c. The disposition and renal handling of enprofylline in endotoxemic rats by bacterial lipopolysaccharide (LPS). Drug Metab. Dispos. 21, 611–616.

Nadai, M., Hasegawa, T., Wang, L., Haghgoo, S., Okasaka, T., Nabeshima, T., Kato, N., 1996. Alterations in renal uptake kinetics of the xanthine derivatives enprofylline in endotoxaemic mice. J. Pharm. Pharmacol. 48, 744–748.

Nadai, M., Sekido, T., Matsuda, I., Wang, L., Kitaichi, K., Itoh, A., Nabeshima, T., Hasegawa, T., 1998. Time-dependent effects of *Klebsiella pneumoniae* endotoxin on hepatic drug-metabolizing enzyme activity in rats. J. Pharm. Pharmacol. 50, 871–879.

Nadai, M., Zhao, Y.L., Wang, L., Nishio, Y., Takagi, K., Kitaichi, K., Takagi, K., Yoshizumi, H., Hasegawa, T., 2001. Endotoxin impairs biliary transport of sparfloxacin and its glucuronide in rats. Eur. J. Pharmacol. 432, 99–105.

Ohnishi, T., Tamai, I., Sakanaka, K., Sakata, A., Yamashima, T., Yamashita, J., Tsuji, A., 1995. In vivo and in vitro evidence for ATP-dependency of P-glycoprotein-mediated efflux of doxorubicin at the blood–brain barrier. Biochem. Pharmacol. 49, 1541–1544.

Rao, V.V., 1999. Choroid plexus epithelial expression of MDR1 P-glycoprotein and multidrug resistance-associated protein contribute to the blood–cerebrospinal fluid drug-permeability barrier. Proc. Natl. Acad. Sci. U. S. A. 96, 3900–39005.

Renton, K.W., Nicholson, T.E., 2000. Hepatic and central nervous system Cytochrome *P*450 are down-regulated during lipopolysaccharide-evoked localized inflammation in brain. J. Pharmacol. Exp. Ther. 294, 524–530.

Renton, K.W., Dibb, S., Levatte, T.L., 1999. Lipopolysaccharide evokes the modulation of brain Cytochrome *P*4501A in the rat. Brain Res. 842, 139–147.

Rousselle, C., Clair, P., Lefaauconnier, J.-M., Kaczorex, M., Schermann, J.-M., Temsamani, J., 2000. New advances in the transport of doxorubicin through the blood–brain barrier by a peptide vector-mediated strategy. Mol. Pharmacol. 57, 679–686.

Saito, T., Zhang, Z.-J., Tokuriki, M., Ohtsubo, T., Shibamori, Y., Yamamoto, T., Saito, H., 2001. Cyclosporin A inhibits the extrusion pump function of P-glycoprotein in the inner ear of mice treated with vinblastine and doxorubicin. Brain Res. 901, 265–270.

Schinkel, A.H., Smith, J.J.M., van Tellingen, O., Wagenaar, E., van Deemter, L., Mol, C.A.A.M., van del 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.

Sewer, M.B., Koop, D.R., Morgan, E.T., 1996. Endotoxemia in rats is associated with induction of the P4504 A subfamily and suppression of several other forms of Cytochrome *P*450. Drug Metab. Dispos. 24, 401–407.

Shukla, A., Dikshit, M., Srimal, R.C., 1995. Nitric oxide modulates blood–brain barrier permeability during infections with an inactivated bacterium. NeuroReport 6, 1629–1632.

Speeg, K.V., Maldonado, A.L., 1994. Effect of the nonimmunosuppressive cyclosporin analog SDZ PSC-833 on colchicine and doxorubicin biliary secretion by the rat in vivo. Cancer Chemother. Pharmacol. 34, 133–136.

Tang, W., Yi, C., Kalitsky, J., Piquette-Miller, M., 2000. Endotoxin down-regulates hepatic expression of P-glycoprotein and MRP2 in 2-acetylaminofluorene-treated rats. Mol. Cell Biol. Res. Commun. 4, 90–97.

Tavoloni, N., Guarino, A.M., 1980. Disposition and metabolism of adriamycin in the rat. Pharmacology 21, 244–255.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I., Willingham, M.C., 1987. Cellular localization of the multidrug-resistant gene product P-glycoprotein in normal human tissue. Proc. Natl. Acad. Sci. U. S. A. 84, 7735–7738.

Tsuji, A., 1998. P-glycoprotein-mediated efflux transport of anticancer drugs at the blood–brain barrier. Ther. Drug Monit. 20, 588–590.

Tsuruo, T., 1988. Mechanisms of multidrug resistance and implications for therapy. Jpn. J. Cancer Res. 79, 285–296.

Tsuruo, T., Iida, H., Tsukagoshi, S., Sakurai, Y., 1982. Increased accumulation of vincristine and Adriamycin in drug-resistant tumor cells following incubation with calcium antagonists and calmodulin inhibitors. Cancer Res. 42, 4730–4733.

Ueda, K., Clark, D.P., Chen, C.J., Roninson, L.B., Gottesman, M.M., Pastan, M.M., 1987. The human multidrug resistance (mdr1) gene cDNA cloning and transcription inhibition. J. Biol. Chem. 262, 505–508.

Van Asperen, J., Van Tellingen, O., Beijnen, J.H., 2000. The role of mdr1a P-glycoprotein in the biliary and intestinal secretion of doxorubicin and vinblastine in mice. Drug Metab. Dispos. 28, 264–267.

Van Bree, J.B.M.M., De Boer, A.G., Danhof, M., Van Ginsel, L.A., Breimer, D.D., 1988. Characterization of an ''in vitro'' blood–brain barrier (BBB): effects of molecular size and lipophilicity on cerebrovascular endothelial transport rates of drugs. J. Pharmacol. Exp. Ther. 247, 1233–1239.

Vos, T.A., Hooiveld, G.J., Koning, H., Childs, S., Meijer, D.K., Moshage, H., Jansen, P.L., Muller, M., 1998. Up-regulation of the multidrug resistance genes, Mrp1 and Mdr1b, and down-regulation of the organic anion transporter, Mrp2, and the bile salt transporter, Spgp, in endotoxemic rat liver. Hepatology 28, 1637–1644.

Wang, L., Hasegawa, T., Nadai, M., Muraoka, I., Nabeshima, T., Kato, N., 1993. The effect of lipopolysaccharide on the disposition of xanthines in rats. J. Pharm. Pharmacol. 45, 34–38.

Warren, K.E., Patel, M.C., McCully, C.M., Montuenga, L.M., Balls, F.M., 2000. Effect of P-glycoprotein modulation with cyclosporin A on cerebrospinal fluid penetration of doxorubicin in non-human primates. Cancer Chemother. Pharmacol. 45, 207–212.

Watchko, J.F., Daood, M.J., Hansen, T.W., 1998. Brain bilirubin content is increased in P-glycoprotein-deficient transgenic null mutant mice. Pediat. Res. 44, 763–766.

Weenen, H., van Maanen, J.M., de Planque, M.M., McVie, J.G., Pinedo, H.M., 1984. Metabolism of 4'-modified analogs of doxorubicin. unique glucuronidation pathway for 4'-epidoxorubicin. Eur. J. Cancer Clin. Oncol. 20, 919–926.

Zhang, Z.-J., Saito, T., Kimura, Y., Sugimoto, C., Ohtsubo, T., Saito, H., 2000. Disruption of mdr1a P-glycoprotein gene results in dysfunctions of blood–inner ear barrier in mice. Brain Res. 852, 116–126.

Zhao, Y.L., Cai, S.H., Wang, L., Kitaichi, K., Tatsumi, Y., Nadai, M., Yoshizumi, H., Takagi, K., Hasegawa, T., 2002. Possible involvement of P-glycoprotein in the biliary excretion of grepafloxacin. Clin. Exp. Pharmacol. Physiol. 29, 167–172.