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Azithromycin reverses anticancer drug resistance and modifies hepatobiliary excretion of doxorubicin in rats

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Abstract

The present study aims to investigate whether azithromycin reverses *P*-glycoprotein-dependent anticancer drug resistance in vitro and modifies the hepatobiliary excretion of doxorubicin, a substrate for *P*-glycoprotein in vivo. Azithromycin increased dose-dependently the intracellular accumulation of doxorubicin in adriamycin-resistant human myelogenous leukemia cells (K562/ADR) with no effect on the expression of *P*-glycoprotein in the cells. However, the inhibitory effect was much weaker than that of cyclosporin A and was comparable to that of erythromycin. When Sprague–Dawley (SD) rats, which have drug transporting *P*-glycoprotein and multidrug resistance-associated protein 2 (Mrp2) in the bile canalicular membrane of hepatocytes, received an infusion of doxorubicin, the steady-state biliary clearance of doxorubicin was significantly decreased for 40 min after a single intravenous injection of azithromycin. However, azithromycin did not increase the plasma concentration of doxorubicin. The biliary clearance of doxorubicin in Eisai hyperbilirubinemic rats (EHBRs), which have a hereditary deficiency in Mrp2, was significantly decreased compared with that in Sprague–Dawley rats, suggesting the involvement of Mrp2 in the biliary excretion of doxorubicin. The present findings suggest that azithromycin overcomes *P*-glycoprotein-dependent anticancer drug resistance of tumors by inhibiting the binding of doxorubicin to *P*-glycoprotein in K562/ADR cells and inhibits the hepatobiliary excretion of drugs that are substrates for *P*-glycoprotein and Mrp2.

Keywords: Azithromycin; *P*-glycoprotein; Multidrug resistance; Doxorubicin; Mrp2 (Multidrug resistance-associated protein 2); Biliary excretion; (Rat)

1. Introduction

P-glycoprotein, a member of the ATP-binding cassette (ABC) transport proteins, is known to efflux hydrophobic and cationic antitumor drugs such as anthracycline antibiotics and *Vinca* alkaloids from anticancer drug resistant cells of tumors (Tsuruo et al., 1982; Tsuruo, 1988; Endicott and Ling, 1989; Sharom et al., 1993). There is evidence that chemical structurally unrelated and clinically used drugs, such as cyclosporin A, verapamil and tacrolimus (FK506), strongly reverse *P*-glycoprotein-dependent anticancer drug resistance (Tsuruo et al., 1981, 1982; Twentyman et al., 1987; Akiyama et al., 1988; Naito et al., 1992). However, these drugs are unsuitable for clinical use as modifiers because of their severe adverse reactions. In a series of our research to develop modifiers for the anticancer drug resistance, we have reported that *N*-ethoxycarbonyl-7-oxo-staurosporine (NA-382), a staurosporine derivative, increases the intracellular accumulation of vinblastine in the mouse leukemia anticancer drug resistant cells P388/ADR and enhances its antitumor activity in vivo (Miyamoto et al., 1993a,b, 1995). Further studies have reported that macrolide antibiotics, erythromycin and clarithromycin, and quinolone antimicrobial agents, sparfloxacin and tosufloxacin, increase the intracellular accumulation of *P*-glycoprotein substrate, doxorubicin, in the P388/ADR cells (Kiso et al., 2000; Wang et al., 2000; Zhao et al., 2002). We consider that macrolide antibiotics might be useful as modifiers for anticancer drug

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resistance since they are one of the safest groups of antimicrobial agents in clinical use and to cause rarely severe adverse reactions.

P-glycoprotein existed not only in anticancer drug resistant cells, but also in a variety of normal tissues such as the liver, kidney, small intestine and capillary endothelium in the brain, and plays a key role in the disposition of hydrophobic and cationic anticancer drugs (Thiebaut et al., 1987; Schinkel et al., 1996, 1997). Like *P*-glycoprotein, multidrug resistance-associated protein 2 (Mrp2) also existed in the same tissues as *P*-glycoprotein (Borst et al., 1999; König et al., 1999). This drug transporting protein also acts as an active efflux pump for a wide range of organic anions such as glutathione, glucuronate and sulfate conjugates by an ATP-dependent mechanism (Oude Elferink et al., 1995). Thus, both drug transporters appear to play a key role in absorption, distribution and elimination of various drugs.

It is reported that erythromycin and clarithromycin, 14-ring member macrolide antibiotics, inhibit *P*-glycoprotein-mediated transport of digoxin, rhodamine 123 and doxorubicin, which are substrates for *P*-glycoprotein (Wakasugi et al., 1998; Yumoto et al., 1999; Kiso et al., 2000). However, these antibiotics are known to inhibit cytochrome *P*450 (CYP) 3A4, which is the most important drug-metabolizing enzyme in the body, and induce many drug interactions based on drug metabolism. On the other hand, azithromycin, a newly developed 15-ring member macrolide antibiotic, is not a substrate for CYP3A4 and mainly eliminated as unchanged form in the feces via biliary excretion and intestinal secretion in humans (Singlas, 1995). Most recently, we have found that azithromycin is excreted into the bile and small intestine via *P*-glycoprotein and Mrp2 in rats (Sugie et al., in press). Azithromycin might delay their hepatobiliary excretion of drugs that are substrates for these transporters and are mainly excreted into the bile in humans. However, there is no information available regarding the drug pharmacokinetic interaction between azithromycin and substrates for these two transporters in humans and reversal effect of azithromycin in *P*-glycoprotein-dependent anticancer drug resistance.

The present study aims to clarify whether azithromycin can reverse *P*-glycoprotein-dependent anticancer drug resistance using human myelogenous leukemia cells (K562/S) and its adriamycin-resistant cells (K562/ADR) and whether it modifies the hepatobiliary excretion of doxorubicin, a substrate for *P*-glycoprotein, in Sprague–Dawley (SD) rats possessing Mrp2 and Eisai hyperbilirubinemic rats (EHBRs) lacking Mrp2.

2. Materials and methods

2.1. Chemicals

Azithromycin was kindly donated from Pfizer Pharm. (Lot. 0A0178, Tokyo, Japan). Doxorubicin hydrochloride and daunorubicin hydrochloride were purchased from Sigma (St. Louis, MO). Doxorubicin hydrochloride, in the form of a commercial preparation for injection used in the in vivo experiment, was purchased from Kyowa Hakko Kogyo (Adriamycin, Tokyo, Japan). Cyclosporin A was purchased from Novartis Pharma (Tokyo, Japan). All other reagents are commercially available and were of analytical grade. Azithromycin was dissolved in 1 M phosphoric acid and the pH was then adjusted to 7.4 with 1 M NaOH. Doxorubicin was dissolved in saline.

2.2. Cell culture

Human chronic myelogenous leukemia cell line, K562/S, and its adriamycin-resistant subline, K562/ADR, were kindly provided by Professor Ken-ichi Miyamoto (Kanazawa University School of Medicine). These cells were routinely cultured in RPMI 1640 medium supplemented with 1% penicillin/streptomycin, 1 mM L-glutamine and 10% heat-inactivated fetal calf serum (Gibco Biocult), at 37 °C in 5% CO_2 humidified atmosphere. Adriamycin (400–500 nM) was added every 2 weeks to the culture medium of K562/ADR cells. The resistant K562/ADR cells were grown for 7 days in the absence of adriamycin prior to use in the experiments. Cell viability was assessed by trypan blue dye exclusion.

2.3. Animals

Male Sprague–Dawley rats (280–295 g body weight) were obtained from Japan SLC (Hamamatsu, Japan). Male Eisai hyperbilirubinemic rats (EHBRs), which have a hereditary deficiency in multidrug resistance associated protein 2 (Mrp2) (Takenaka et al., 1995; Yamazaki et al., 1996), weighing 285–305 g, were also obtained from the same company as described above. The rats were housed under controlled environmental conditions (temperature of 23 ± 1 °C and humidity of 55 ± 5%) with a commercial food diet and water freely available to animals. 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.

2.4. Intracellular uptake experiments

K562/S and K562/ADR cells (2×10^6 cells/ml) were suspended in phosphate buffered saline (PBS) solution (pH 7.2), and were incubated with doxorubicin (5 μM) in the presence or absence of azithromycin for 2 h at 4 or 37 °C because the maximum uptake occurred at 120 min. The number of cells was determined by hemocytometer. After incubation, the cells were washed three times with cold PBS solution and the obtained cell pellets were kept at − 30 °C until analysis. For the measurement of doxorubicin in cells, the pellets were suspended in 50 μl of water containing daunorubicin (1.8 μg/ml) as an internal standard, and then

ultrasonicated with an ultrasonic disrupter (UD-2000, Tomy Seiko Tokyo, Japan). Acetonitrile was added to the ultrasonicated solution and was shaken vigorously. After centrifugation by 3000 × *g* at 4 °C for 5 min, acetonitrile layer was collected into the glass tubes and was evaporated under a nitrogen gas stream at 45 °C. The residue was reconstituted with the mobile phase to measure doxorubicin by high-performance liquid chromatography (HPLC).

2.5. Hepatobiliary clearance experiments

To investigate whether azithromycin modifies the biliary excretion of doxorubicin, rats under anesthesia with sodium pentobarbital (40 mg/kg) were cannulated with polyethylene tubes into the carotid artery, the jugular vein and the bile duct. The rats received a loading dose of 0.26 mg/kg and a maintenance dose of 156 μg/h at a rate of 2 ml/h until the end of the study. The loading and maintenance doses of doxorubicin used in this study referred to our previous study (Hidemura et al., 2003). After a 60-min infusion, bile samples were collected at 20-min interval for 60 min. Blood samples were collected at the midpoints of the bile collection periods (70, 90 and 110 min after the infusion was started). After a 120-min infusion, azithromycin (60 mg/kg) was administered intravenously. Bile samples were collected at 20-min interval from 120 to 220 min. Blood samples were collected at the midpoints of the bile collection periods (130, 150, 170, and 190 min after the infusion was started). The volume of bile samples was measured gravimetrically with specific gravity assumed to be 1.0. This experiment was done under anesthesia with pentobarbital, and the body temperatures of the animals were maintained at 37 °C with a heat lamp.

2.6. Western blot analysis

K562/S and K562/ADR cells were suspended in 1 ml of 20 mM HEPES–NaOH buffer (pH 7.2) containing 1% Triton X-100, and were incubated at 37 °C for 10 min. The suspension was centrifuged at 80,000 × *g* for 60 min at 4 °C. The pellet was dissolved in 1 ml of 10 mM Tris–HCl buffer (pH 8.0) containing 0.5% Nonidet P40 (Daiichi Kagaku Yakuhin, Tokyo, Japan). The protein concentration in the solution was measured with Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin (Sigma) as a standard.

The protein (20 μg) was separated by electrophoresis on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked in PBS solution containing 0.1% Tween 20 and 4% nonfat dry milk, detected by C219 mouse monoclonal antibody (DAKO, Glostrup, Denmark) to *P*-glycoprotein and human monoclonal antibody against MRP2 (Alexis Biochemicals, San Diego, CA). Quantification of the band intensity was performed using NIH image program (National Institutes of Health, Bethesda, MD).

2.7. Drug analysis

Concentrations of doxorubicin in plasma and bile were determined by HPLC. Bile samples were properly diluted in distilled water. Briefly, 50 μl of each sample and 300 μl of methanol containing an internal standard of daunorubicin (0.2 μg/ml) were mixed and centrifuged at 6000 × *g* for 10 min. After centrifugation, the supernatant (100 μl) was subjected directly to HPLC. The apparatus used for HPLC was Shimadzu LC-10A system (Kyoto, Japan) equipped with a fluorescence detector (RF-10AXL, Shimadzu) (excitation, 480 nm; emission, 560 nm) consisting of an LC-10A liquid pump and an SIL-10A autoinjector. The conditions were as follows: column, a Cosmocil $5C_{18}$ column (4.6 by 150 mm, Nacalai Tesque, Kyoto, Japan); mobile phase, 10 mM phosphate buffer (pH 4.0)–methanol (40:60 [vol/vol]) solution; column temperature (CTO-10AC, Shimadzu), 50 °C; flow rate, 1.2 ml/min. This assay was shown to be linear for the concentrations with a correlation coefficient of 0.999. No interference with the peak of doxorubicin was observed in any samples. The detection limit was approximately 0.02 μg/ml for plasma and bile samples. The within-day and between-day coefficients of variation for this assay were less than 8%.

2.8. Data analysis

The biliary clearance (CL_{BILE}) was calculated by dividing the respective excretion rates by steady-state plasma concentration (C_{SS}) determined for that collection period.

2.9. Statistical analysis

The results are expressed as means ± S.E. for the indicated numbers of experiments. Student's *t*-test was used to assess the statistical significance of differences. A confidence level of <0.05 was considered as statistically significant.

3. Results

3.1. In vitro accumulation of doxorubicin in K562/S and K562/ADR cells

We confirmed whether *P*-glycoprotein and/or Mrp2 are expressed in K562/ADR cells by Western blot analysis. As shown in Fig. 1, *P*-glycoprotein was overexpressed in K562/ADR, but not in the K562/S cells. No expression of Mrp2 was observed in either K562/S cells or K562/ADR cells (data not shown). In addition, neither azithromycin alone nor azithromycin and doxorubicin had effect on the protein level of *P*-glycoprotein in K562/ADR cells.

The time-course of the intracellular accumulation of doxorubicin into K562/S and K562/ADR cells are shown in Fig. 2. The concentration of doxorubicin accumulated in

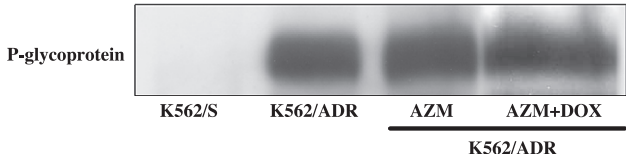

Fig. 1. A representative Western blot of *P*-glycoprotein in K562/S cells or K562/ADR cells in the presence of azithromycin (AZM) or azithromycin and doxorubicin (DOX). K562/ADR cells were incubated with AZM (1 mM) or AZM plus DOX (1 mM and 5 μM).

both cells was higher at 37 °C than 4 °C and the amount of doxorubicin accumulated in K562/S cells at 120 min was approximately 5-fold higher than that in K562/ADR cells, indicating that doxorubicin is effectively pumped out of K562/ADR cells via *P*-glycoprotein.

3.2. Effect of azithromycin on intracellular accumulation of doxorubicin

Fig. 3 shows the effects of azithromycin, erythromycin and cyclosporin A on the intracellular accumulation of doxorubicin in K562/S and K562/ADR cells. As shown in Fig. 3, the accumulation of doxorubicin by K562/S cells, incubated with 5 μM of doxorubicin at 37 °C for 120 min, was not influenced by the presence of 1 mM of azithromycin. In contrast, the intracellular accumulation of doxorubicin into K562/ADR cells in the presence of 1 mM of azithromycin was approximately 1.5-fold of that in the absence of azithromycin (Fig. 3). On the other hand, cyclosporin A (10 μM) significantly increased the accumulation of doxorubicin, suggesting that cyclosporin A has a stronger binding potency to *P*-glycoprotein than azithromycin.

3.3. Inhibitory effect of azithromycin against biliary excretion of doxorubicin in SD rats and EHBRs

To clarify whether azithromycin inhibits *P*-glycoprotein- and Mrp2-mediated biliary excretion of doxorubicin, in vivo biliary clearance experiments were performed in SD rats. The control values of the biliary clearance and steady-state

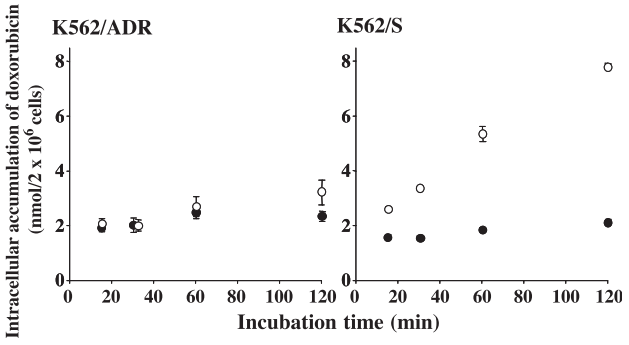

Fig. 2. Time-dependent intracellular accumulation of doxorubicin in K562/ADR and K562/S cells at 4 °C (●) or 37 °C (○). Values are the mean ± S.E.M. of three experiments (when the S.E.M. is small, it is included in the symbol).

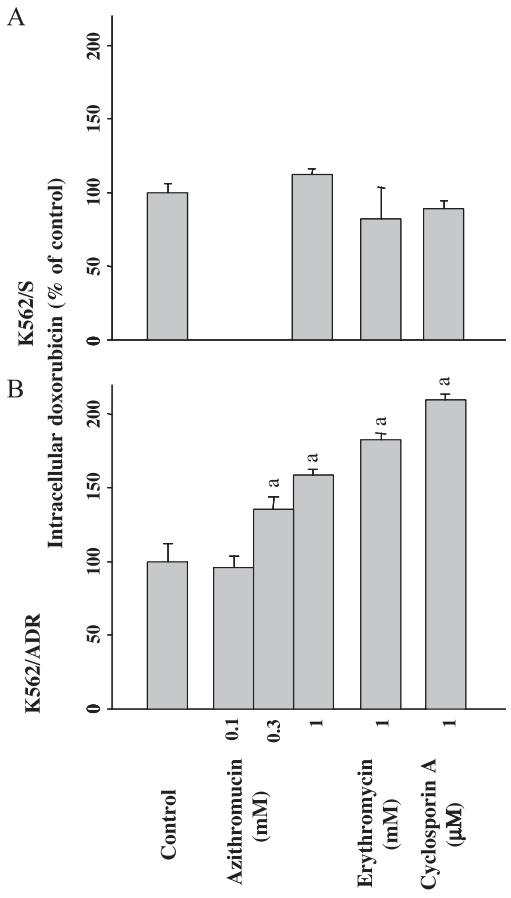

Fig. 3. Effect of azithromycin (0.1, 0.3 and 1 mM), erythromycin (1 mM) and cyclosporin A (1 μM) on the uptake of doxorubicin in (A) K562/ADR and (B) K562/S cells. Data are the mean ± S.E.M. of three experiments. [a]Significantly different from the control ($P < 0.05$).

plasma concentration of doxorubicin in SD rats were 5.20 ± 0.48 ml/min and 0.19 ± 0.01 μg/ml, respectively. As shown in Fig. 4, azithromycin significantly decreased

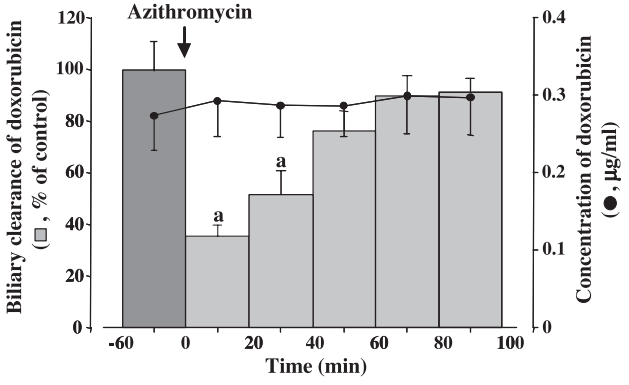

Fig. 4. Effect of azithromycin (60 mg/kg) on the biliary clearance (■) and steady-state plasma concentration (●) of doxorubicin in SD rats. The mean control biliary clearance of doxorubicin (−60 to 0 min) was 5.20 ± 0.48 ml/min. The mean steady-state control plasma concentration of doxorubicin was 0.19 ± 0.01 μg/ml. Data are the mean ± S.E.M. of five rats. At the time point indicated by the arrow, azithromycin was administered intravenously. [a]Significantly different from the control value ($P < 0.05$).

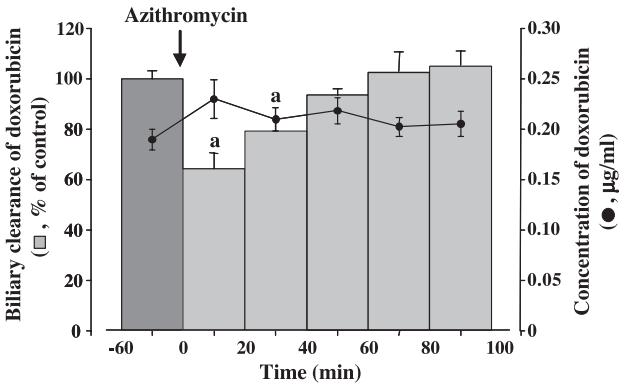

Fig. 5. Effect of azithromycin (60 mg/kg) on the biliary clearance (■) and steady-state plasma concentration (●) of doxorubicin in EHBRs. The mean control biliary clearance of doxorubicin (− 60 to 0 min) was 2.44 ± 0.44 ml/min. The mean steady-state control plasma concentration of doxorubicin was 0.27 ± 0.04 μg/ml. Data are the mean ± S.E.M. of five rats. At the time point indicated by the arrow, azithromycin was administered intravenously. [a]Significantly different from the control value ($P < 0.05$).

the biliary clearance of doxorubicin by 35% during zero to 20 min and 20% during 20 to 40 min after injection, compared with the control value (− 60 to 0 min). However, azithromycin injection did not change the plasma concentration of doxorubicin.

The control values of the biliary clearance and steady-state plasma concentration of doxorubicin in EHBRs were 2.44 ± 0.44 ml/min and 0.27 ± 0.04 μg/ml, respectively. The biliary clearance of doxorubicin in EHBRs was decreased to approximately 50% of that in SD rats. The mean plasma concentration of doxorubicin in EHBRs was significantly higher than that in SD rats. As shown in Fig. 5, azithromycin significantly decreased the biliary clearance of doxorubicin by 65% during zero to 20 min and 50% during 20 to 40min after injection compared with the control value (− 60 to 0 min). However, azithromycin did not change the plasma concentration of doxorubicin.

4. Discussion

It is known that modifiers of *P*-glycoprotein-dependent anticancer drug resistance increase the amount of anticancer drugs in anticancer drug resistant cells by binding to the intracellular or transcellular domains of *P*-glycoprotein or by inducing conformational changes in *P*-glycoprotein, thereby enhancing the anticancer effect by increasing the cytotoxicity of the anticancer drugs (Akiyama et al., 1988; Cornwell et al., 1987; Yusa and Tsuruo, 1989; Wakusawa et al., 1992; Zordan-Nudo et al., 1993; Pawagi et al., 1994). Numerous modifiers that overcome *P*-glycoprotein-related anticancer drug resistance have been introduced. In specially, the potent modifiers cyclosporin A and FK506, which are used clinically to protect against graft rejection after organ transplantation, based on their immunosuppressive effects, have the undesirable effects such as renal failure. Contrary to these drugs, macrolide antibiotics might make a clinically safe modifier since they have reversal effect on *P*-glycoprotein-dependent anticancer drug resistance (Wang et al., 2000) and are widely used to prevent bacterial infections in patients and rarely cause serious untoward effects.

In the first in vitro uptake experiments, we examined the effect of azithromycin, which is a new 15-ring member macrolide antibiotic, on the efflux of doxorubicin from K562/S cells not possessing *P*-glycoprotein and K562/ADR cells possessing *P*-glycoprotein. The in vitro experiments showed that azithromycin significantly increased the intracellular accumulation of doxorubicin in K562/ADR cells, but had no effect on that in K562/S cells and did not change the protein level of *P*-glycoprotein in K562/ADR cells. It is unlikely that the increased accumulation of doxorubicin in the presence of azithromycin is due to azithromycin-induced down-regulation of *P*-glycoprotein in K562/ADR cells. The inhibitory potency of azithromycin appears to be similar to that of erythromycin, probably due to their minor structural differences. It is likely that the chemical structure of macrolide antibiotics plays a key role in the inhibitory activity of *P*-glycoprotein. These results suggest that azithromycin can reverse anticancer drug resistance, although the potency is much weaker than cyclosporin A, which are supported by our previous studies that macrolide antibiotics overcome anticancer drug resistance (Wang et al., 2000).

Considering that anticancer chemotherapy may be accompanied with antibiotic therapy including azithromycin and that *P*-glycoprotein is existed in the bile canalicular membrane of hepatocytes in animals and humans and plays an important role in the hepatobiliary excretion of drugs (Thiebaut et al., 1987), the effect of azithromycin on *P*-glycoprotein-mediated excretion of anticancer drugs should be investigated. In the second of in vivo experiments in SD rats, we investigated whether azithromycin can modify *P*-glycoprotein and/or Mrp2-mediated hepatobiliary excretion of doxorubicin since we have found that azithromycin is not secreted into the urine by these transporters (Sugie et al., in press). The in vivo biliary clearance experiments showed that azithromycin significantly decreased the biliary clearance of doxorubicin for 40 min after injection, suggesting that azithromycin inhibits hepatobiliary *P*-glycoprotein and/or Mrp2. We previously reported that the biliary clearance of doxorubicin was almost completely inhibited by injection of erythromycin in Wistar rats (Kiso et al., 2000). In the present study in SD rats, azithromycin was found to have a much weaker inhibitory effect of hepatobiliary excretion of doxorubicin than erythromycin, although the in vitro inhibitory effect of azithromycin and erythromycin on the efflux of doxorubicin from K562/ADR cells was almost the same. We have found that the percentage of azithromycin excreted into the bile is approximately 1% of dose administered in SD rats (Sugie et al., in press). The discrepancy between azithromycin and erythromycin may be due to the strain-related difference. Other possible explanation is that the

percentage of azithromycin excreted into the bile is lower than that of erythromycin, although there is no report about erythromycin.

It is reported that the substrate specificities of *P*-glycoprotein, CYP3A4 and Mrp2 overlap (Mayer et al., 1995; Oude Elferink et al., 1995; Wacher et al., 1995) and that the sensitivity of cells to doxorubicin also correlates with the expression of Mrp2 (Cui et al., 1999; Koike et al., 1997), suggesting that doxorubicin is a substrate for Mrp2. It is possible that a decreased biliary excretion of doxorubicin by azithromycin found in SD rats is due to the inhibition of both *P*-glycoprotein and Mrp2. To our knowledge, there is no in vivo evidence for Mrp2-mediated biliary transport of doxorubicin. We, therefore, examined the effect of azithromycin on the hepatobiliary excretion of doxorubicin in EHBRs not possessing Mrp2. The mean control value of the biliary clearance of doxorubicin obtained in EHBRs (2.44 ml/min) was approximately 50% of that in SD rats (5.20 ml/min). These results suggest that the decreased biliary excretion of doxorubicin in EHBRs is due to deficiency of Mrp2 in the bile canalicular membrane hepatocytes and that doxorubicin is excreted into the bile via both *P*-glycoprotein and Mrp2. In EHBRs, azithromycin significantly decreased the biliary clearance of doxorubicin by 65%, suggesting that azithromycin inhibits *P*-glycoprotein. On the basis of these findings, it is most likely that the inhibitory potency of Mrp2 by the dose of azithromycin used in this study was much weaker than that of *P*-glycoprotein by azithromycin. It was also confirmed that doxorubicin is excreted into the bile by the drug transporting Mrp2 and *P*-glycoprotein.

In conclusion, the present study is the first to report that azithromycin, which is not a substrate for CYP3A4, inhibited both the in vitro efflux of doxorubicin from K562/ADR cells overexpressing *P*-glycoprotein and the in vivo biliary excretion of doxorubicin by the drug transporters *P*-glycoprotein and Mrp2 without marked increase in the plasma concentrations. The results reported here, at least, should provide further information that chemotherapy by doxorubicin in combination with azithromycin could be useful in overcoming the *P*-glycoprotein- and/or Mrp2-mediated multidrug resistance of tumors.

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