Quantitative Investigation of the Impact of P-glycoprotein Inhibition on Drug Transport across Blood-Brain Barrier in Rats

Hiroshi Sugimoto, Hideki Hirabayashi, Yoshiaki Kimura, Atsutoshi Furuta, Nobuyuki Amano, and Toshiya Moriwaki

Running title: Impact of the P-gp-mediated drug interaction at the rat BBB

Corresponding author: Hiroshi Sugimoto

Discovery Research Center, Pharmaceutical Research Division
Takeda Pharmaceutical Company Limited
2-17-85 Juso-Honmachi, Yodogawa-ku, Osaka 532-8686, Japan
Phone: (+81) 6-6308-9071
Fax: (+81) 6-6308-9019
E-mail: Sugimoto_Hiroshi1@takeda.co.jp

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Abbreviations:
P-gp, P-glycoprotein; BBB, blood-brain barrier; IVIVC, in vitro-in vivo correlation; MCFR, modified corrected flux ratio; $K_{p,\text{brain}}$, brain-to-plasma concentration ratio; DDI, drug-drug interaction; ABC, ATP-binding cassette; FR, flux ratio; CFR, corrected flux ratio; LY, lucifer yellow; UFLC, ultra-fast liquid chromatography; LC/MS/MS, liquid chromatography with triple quad mass spectrometric detection
Abstract

The magnitude of P-glycoprotein (P-gp)-mediated drug-drug interaction (DDI) at the blood-brain barrier (BBB) in rats was estimated by in vitro-in vivo correlation (IVIVC). In in vitro studies, rat Mdr1a-expressing LLC-PK1 cells were examined for the evaluation of P-gp inhibitory activity using digoxin as a P-gp probe substrate. The in vitro Ki value was calculated using modified corrected flux ratio (MCFR), which reflects the P-gp function. In in vivo studies, digoxin with or without P-gp inhibitors was administered to rats by constant intravenous infusion in order to evaluate the effect of P-gp inhibition on the digoxin transport to the brain under steady-state conditions. In the presence of elacridar, the brain-to-plasma concentration ratio ($K_{p,brain}$) of digoxin was approximately 14 times of the control value. However, no significant change in the $K_{p,brain}$ was observed in the presence of clinically used P-gp inhibitors except cyclosporin A. A positive correlation was found between the in vivo $K_{p,brain}$ of digoxin and $[I_{unbound}/K]$ ($I_{unbound}$: unbound plasma concentration of P-gp inhibitors). Compounds with $[I_{unbound}/K] > 1$ increased $K_{p,brain}$ of digoxin in rats. In summary, we quantitatively evaluated the impact of P-gp-mediated DDI at the rat BBB. We successfully established the IVIVC, which indicated the potential DDI in the presence of potent P-gp inhibitors. On the basis of the IVIVC in rats and Ki values in human MDR1, we speculated that clinically used P-gp inhibitors do not cause DDI at the human BBB, as none of the compounds studied showed $[I_{unbound}/K] > 1$ at therapeutic doses.
Introduction

P-glycoprotein (P-gp, ABCB1) is a member of ATP-binding cassette (ABC) efflux transporters, and it can have a significant effect on drug absorption, distribution, and excretion of a number of clinically relevant drugs. P-gp also functions at the blood-brain barrier (BBB) and facilitates the excretion of potentially toxic xenobiotics (Tsuji et al., 1992). Understandings the relevance of P-gp-mediated drug-drug interaction (DDI) has become important for drug discovery and development. This is because it has been found that in mice, the central nerve system penetrations of several drugs, such as vinblastine, digoxin, cyclosporin A, and dexamethasone is regulated by P-gp (Schinkel et al., 1994; Schinkel et al., 1995). Recently, in vitro P-gp inhibition assays (Keogh and Kunta, 2006) and in vivo positron emission tomography (PET) studies in humans have been conducted to predict the possibility of DDI at the BBB (Hsiao et al., 2006; Hsiao et al., 2008).

However, despite significant efforts to predict the effect of P-gp inhibition, a quantitative approach without any influence of metabolism and other transporter has not yet been carried out. Additionally, there is very limited information that shows whether P-gp-related DDI at the human BBB is observed at the therapeutic blood concentrations of P-gp inhibitors. To address these issues, we aimed to clarify the impact of P-gp inhibition on the drug transport across the BBB by conducting quantitative evaluation of P-gp inhibition and in vitro-in vivo correlation (IVIVC) in rats.

For a theoretical interpretation of P-gp-mediated DDI, it is necessary to set an appropriate protocol for in vitro P-gp inhibition assay using a P-gp-specific probe substrate. A number of methods have been developed for estimating the magnitude of P-gp inhibition. (Adachi et al., 2001; Hsiao et al., 2006; Keogh and Kunta, 2006). Adachi et al. demonstrated that the corrected flux ratio (CFR), which is ascertained by dividing the flux ratio (FR) of basal-to-apical and apical-to-basal transport across human MDR1-expressing LLC-PK1 cells by the corresponding FR in mock cells, may be a good predictor for the in vivo P-gp function at the mouse BBB. However, the tight junctions formed in mouse Mdr1a or human MDR1-expressing LLC-PK1 cells are often different from those formed in mock cells. This may cause variations in the passive permeability level among different cell lines. Therefore, we used the modified corrected flux ratio (MCFR), which is the ratio
of the FR across the MDR1-expressing LLC-PK1 cells and the corresponding FR in cells in which the function of P-gp is completely inhibited using a potent inhibitor. On the basis of the MCFR, we estimated the \( K_i \) values using digoxin as a representative P-gp probe substrate.

The use of Mdr1a/1b\((-/-)\) mice and chemically induced P-gp knockout animals for investigating the importance of P-gp at the BBB has been reported in previous studies (Schinkel et al., 1994; Schinkel et al., 1995; Cutler et al., 2006). Traditionally, gene knockout mice have been used for the functional evaluation of transporters. However, the advantage of chemically induced P-gp knockout animals is that any species relevant to the pharmacological model can be employed to examine the effect of P-gp on drug efficacy and dose response. Further, when using the aforementioned P-gp knockout animals, the drug dosage can be suitably adjusted to quantify the magnitude of P-gp inhibition by elacridar or clinically used P-gp inhibitors. We therefore employed P-gp inhibitor-treated rats in this study.

Establishment of the IVIVC regarding digoxin DDI is one of the typical approaches to profile the potential of drug candidates to inhibit P-gp in clinical applications. Recently, the International Transporter Consortium released a regulatory viewpoint on P-gp-mediated DDI, suggesting that the clinical digoxin DDI study would be recommended where the number of \( \frac{[I_{unbound}]}{IC_{50}} \) value (\( I_{unbound} \): unbound concentration of P-gp inhibitors under steady-state conditions) is larger than 0.1 (Giacomini et al., 2010). However, in this case, it is necessary to take into account the large variations in \( IC_{50} \) values, which are attributed to the difference in monolayer cells (i.e. Caco-2, MDR1-LLC/PK1, and MDR1-MDCKII) and parameters used (i.e. basal-to-apical, flux ratio, and CFR). In the present study, we have quantitatively investigated the impact of P-gp inhibition on digoxin transport across the rat BBB using the ratio \( \frac{I_{unbound}}{K_i} \). We set the cutoff values to minimize the effect of potential digoxin-based DDIs on the IVIVC in rats. Finally, by comparing the \textit{in vitro} \( K_i \) values in human MDR1-expressing cells and conventional therapeutic concentrations of P-gp inhibitors, the possibility of the DDI at the human BBB at clinical doses was discussed.
Materials and Methods

Chemicals. Digoxin and verapamil were purchased from Sigma-Aldrich, Co. (MO, USA). Elacridar and cyclosporin A were from Toronto Research Chemicals, Inc (North York, Canada). Itraconazole was from MP Biomedicals, LLC (Tokyo, Japan). Ketococonazole and quinidine were from Wako Pure Chemicals Industries (Osaka, Japan). All other chemicals were the highest reagent grade available from commercial sources.

Cell culture. The rat Mdr1a and human MDR1-expressing LLC-PK1 cells were cultured using a method obtained by slight modification of that reported in the literature (Takeuchi et al., 2006). Briefly, cells were incubated in complete culture medium consisting M199 (Invitrogen, Carlsbad, CA) or DMEM (Dulbecco’s modified Eagle’s medium: Invitrogen) with 20% fetal bovine serum (Invitrogen), 500 μg/mL G418 and 150 ng/mL colchicine. Cells were split bi-weekly at a ratio of 1:5-10 and grown in an atmosphere of 5%CO₂-95%O₂ at 37ºC. Rat Mdr1a- and human MDR1-expressing LLC-PK1 cells were harvested with trypsin and seeded on microporous polycarbonate membrane filters at a cell density of 2.25×10⁵ cells/mL in 24-well insert plates (TranswellTM, 3397, Corning, Acton, MA) and 2.25×10⁶ cells/mL in 96-well insert plates (TranswellTM, 3391, Corning), respectively. These plates were incubated in an atmosphere of 5%CO₂-95%O₂ at 37ºC. Fresh medium was added on the third or fourth day and transcellular transport study was taken place on the seventh day after seeding.

Transcellular transport study. Before the start of the transcellular transport study, all culture media were replaced with the fresh M199 medium including 10 mmol/L HEPES at pH 7.4. Inhibitory effects on vectorial transport of digoxin were investigated by adding P-gp inhibitors to the medium on both sides of the transwell chamber for 30 min before adding substrates. The medium on the donor side of the transwell chamber was replaced with M199 medium including 10 mmol/L HEPES, inhibitors and 10, 20, 50 and 250 μmol/L of digoxin. The transwell of rat Mdr1a- and human MDR1-expressing LLC-PK1 cells were then incubated in an atmosphere of
5% CO₂-95% O₂ at 37°C for 1 or 2 h, respectively. Lucifer yellow (LY) (200 μmol/L) was used in every transwell along with digoxin and inhibitors to monitor the monolayer integrity. Aliquots from the receiver side were taken at time where the linearity of the transcellular transport of digoxin was observed. The LY concentration in the receiver chamber was determined with fluorescence intensity. Excitation and emission wavelengths were set as 485 and 528 nm. The permeability coefficient of LY should be less than 15 nm/sec for the acceptable assay condition.

**Data analysis for the transcellular transport study.** The permeability coefficient of digoxin ($P_{app,A\rightarrow B}$ and $P_{app,B\rightarrow A}$) was calculated according to equation 1 and the efflux ratio was calculated using equation 2.

\[
P_{app} = \frac{\text{Amount}}{\text{Area} \cdot C_0 \cdot \text{Time}}
\]

\[
\text{Efflux ratio} = \frac{P_{app,B\rightarrow A}}{P_{app,A\rightarrow B}}
\]

**Amount:** amount of transported digoxin per well of the cell monolayer  
**Area:** surface area of the cell monolayer (0.143 cm² in 96-well, 0.33 cm² in 24-well)  
**C₀:** initial concentration of digoxin in the donor side  
**Time:** incubation time  

\[P_{app,A\rightarrow B} : \text{AtoB passive permeability-surface area product}\]  
\[P_{app,B\rightarrow A} : \text{BtoA passive permeability-surface area product}\]

The modified corrected flux ratio (MCFR) was defined as the flux ratio in MDR1-expressing LLC-PK1 cells divided by the corresponding ratio in functionally MDR1-inhibited cells using a potent P-gp inhibitor, elacridar. According to the compartment model shown in Fig.1, MCFR across MDR1-expressing cells/MDR1-inhibited cells can be determined using equation 4.

\[
\text{MCFR} = \frac{\text{Efflux ratio in MDR1 cells}}{\text{Efflux ratio in MDR1-inhibited cells}}
\]

\[
= \frac{\frac{\text{PS}_{B,\text{inf}} \times (\text{PS}_{A,\text{eff}} + \text{PS}_{\text{P-gp}})}{\text{PS}_{A,\text{inf}} \times \text{PS}_{B,\text{eff}}}}{1 + \frac{\text{PS}_{\text{P-gp}}}{\text{PS}_{A,\text{eff}}}}
\]
PS_{A,in}, PS_{A,eff}: permeability coefficient for the influx or efflux transport across the apical membrane,

PS_{B,in}, PS_{B,eff}: permeability coefficient for the influx or efflux transport across the basal membrane

PS_{P-gp}: permeability coefficient for the efflux transport mediated by P-gp across the apical membrane

*In vitro* Ki values were calculated based on Dixon plot using the parameter shown in equation 5. The intersection of Dixon plot, which indicates Ki values, was fitted with nonlinear least-squares method using the MULTI program (Yamaoka et al., 1981).

\[
[MCRF-1] \times \text{[initial concentration of digoxin]} = \frac{PS_{P-gp}}{PS_{A,eff}} \times \text{[initial concentration of digoxin]} \quad (5)
\]

**Experimental Animals.** Male Crl:CD(SD) rats (8~9 weeks, 296 g~351 g) were purchased from Charles River Japan Inc. (Shiga, Japan) and housed at controlled temperature and humidity with a 12-h light/dark cycle. Rats received a standard diet and water *ad libitum*. All animal experiments were conducted in accordance with the guideline of Experimental Animal Care and Use Committee of Takeda Pharmaceutical Co. Ltd.

**Intravenous infusion of digoxin in the presence or absence of P-gp inhibitors in rats.** Rats were placed under anesthesia with diethyl ether, the femoral vein was cannulated with a 3.0-Fr (ID: 0.58 mm, OD: 0.94 mm) catheter. Rats were attached to the free-moving system (Eicom Inc., Kyoto, Japan, Tsumura Inc., Tokyo, Japan) during experiments. The catheter was inserted through an incision in the cervical division to attach the free-moving system. Digoxin (0.06 mg/h/kg) was administered by a constant rate intravenous infusion via the femoral vein to achieve steady-state plasma concentration in the presence or absence of P-gp inhibitors. *N*-methyl-2- pyrrolidone (NMP)/saline (1:1) and dimethylacetamide (DMA)/saline (1:1) mixtures were used as dosing vehicle for elacridar and other P-gp inhibitors, respectively. Elacridar (0.125, 0.25, 0.5, 1, 2
mg/h/kg) was concomitantly administered with digoxin for 8 h. Plasma samples were collected from tail vein at 4, 6, and 8 h after the initiation of administration. Itraconazole (0.01, 0.03 mg/h/kg), cyclosporin A (1, 3, 10 mg/h/kg), ketoconazole (1, 3, 10 mg/h/kg), verapamil (1, 3 mg/h/kg), quinidine (1, 3, 10 mg/h/kg) were also concomitantly administered with digoxin for 16 h to achieve pseudo steady-state plasma concentrations. Plasma samples were collected from tail vein at 12, 14, and 16 h after the initiation of administration. The animal was sacrificed by exsanguination at 6 or 16 h, and the brain was harvested. Plasma and brain concentrations were determined with an ultra-fast liquid chromatography (UFLC) connected to a tandem mass spectrometer analytical system (LC/MS/MS). Plasma and 20% brain homogenate samples were frozen at -80 °C until further analysis.

Determination of plasma protein binding.

The unbound fraction in rat and human plasma was determined using the 96-well equilibrium dialysis apparatus (HTDialysis, LLC, Connecticut, USA). The control rat plasma was pooled and kept at -80°C until the plasma protein binding experiment. The control pooled human plasma was purchased from KAC (Kyoto, Japan). The tested compounds was added into the control plasma (the final concentration: 0.1 or 1 μmol/L), and 150 μL of aliquots were subsequently added to the donor chamber of equilibrium dialysis apparatus divided by the dialysis membrane (Single strip HTD96a/b 1103-50, LLC, Connecticut, USA). The receiver chamber was loaded with 150 μL of 50 mmol/L phosphate buffer saline, pH 7.4. The apparatus was placed on the shaker (87 rpm) (Type NR-1, TAITEC, Tokyo, Japan) for 20 h at the room temperature. Previous in-house experiments revealed that equilibrium was adequately achieved within the incubation period for 20 h (data not shown). After incubation, aliquots (50 μL) from donor and receiver side were mixed with 50 μL aliquots of the dialyzed control buffer or human plasma to yield the identical matrix for consistency in quantification. As an internal standard, 150 μL acetonitrile containing 100 ng/mL alprenolol was added to all samples and vigorously mixed, and centrifuged at 4°C and 5250 rpm for 5 minutes. The supernatant was diluted with 0.2% (v/v) formic acid in 10 mmol/L ammonium formate (pH 3), and
aliquot concentrations were determined using a MS/MS apparatus equipped with a UFLC. The unbound fraction (fu, plasma) was calculated from the ratio of concentrations in receiver and donor side of dialysate.

**Quantification.** Samples from the transcellular transport study were mixed with an equal volume of acetonitrile including 100 nmol/L of digoxin-d3 (internal standard) and centrifuged at 4°C, 5250 rpm for 5 min. The supernatant was diluted with an equal volume of 0.1% formic acid solution and centrifuged at 4°C, 5250 rpm for 5 minutes, and the concentration of digoxin in the supernatant was determined by a MS/MS apparatus (API4000, Applied Biosystems, Foster City, USA) equipped with a UFLC (Shimadzu, Kyoto, Japan). The analytical column was Atlantis T3, C18 column, (20 mm × 2.1 mm, 3 μm) (Waters). The total run time, flow rate and column temperature were 1 min, 1 mL/min, and 50 °C, respectively. Mobile phase A and B consisted of 0.2% formic acid and 0.2% formic acid in methanol, respectively. The initial concentration of mobile phase B was 5%; the condition was maintained for 0.02 min, followed by a linear increase of B to 95% over 0.03 min and sustained for 0.45 min. The condition was then returned to the initial concentration and held for 0.5 min for re-equilibration. Detailed MS conditions were shown in Table 1. The lower limit of quantification was 4.6 nmol/L, and the standard curve was linear ($r^2 > 0.99$) over the digoxin concentration range of 4.6-10000 nmol/L.

Digoxin concentrations in plasma and brain determined using a MS/MS apparatus (API5000) equipped with a UFLC. The quantification method used in this study was a slight modification of a previous report (Hirabayashi et al 2010). Briefly, 50 μL of plasma and 20% brain homogenate samples were mixed with 200 μL of acetonitrile, 50 μL of NaCl-saturated 0.1 mol/L sodium hydrate, and 10 μL of lanatoside-C as an internal standard (1 μg/mL) and then centrifuged at 4 °C, 5250 rpm for 5 min. The supernatant was diluted with 2 volumes of distilled water, and digoxin concentration was determined. The analytical column used was a Capcell Pak, C18 column, MGH (20 mm × 2.0 mm i.d., 5 μm) from Shiseido Co. Ltd. (Kanagawa, Japan). The total run time, flow rate and column temperature were 5 min, 0.4 mL/min, and 40 °C, respectively. The mobile phase A and B consisted
of 0.05% (w/v) ammonium carbonate, pH 9 and methanol, respectively. The initial concentration of mobile phase B was 10%, and this condition was maintained for 0.2 min, followed by a linear increase of B to 95% over 2.8 min, held for 0.8 min. The condition was then returned to the initial concentration and held for 1.2 min for re-equilibration. The lower limit of quantification of digoxin in rat plasma and brain were 1 ng/mL and 5 ng/g brain, respectively. The standard curve was linear (r² > 0.999) over the digoxin concentrations of 1-300 ng/mL (rat plasma) and 5-1500 ng/g (rat brain).

Plasma concentrations of P-gp inhibitors were determined using a MS/MS apparatus (API4000) equipped with a UFLC. Briefly, 50 μL of plasma sample and 20% brain homogenate sample were mixed 200 μL of acetonitrile and 10 μL of alprenolol (1 μg/mL) as an internal standard, followed by centrifugation at 4 °C, 5250 rpm for 5 min. The supernatant was diluted with 3 volumes of 10 mmol/L ammonium formate, and drug concentrations were determined. The analytical column used was Shim-pack XR-ODS, C18 column (20 mm × 2.0 mm, 5 μm) from Shimadzu Co. Ltd. (Kyoto, Japan). The total run time, flow rate and column temperature were 2.6 min, 0.5 mL/min, and 50 °C, respectively. Mobile phase A and B consisted of 10 mmol/L ammonium formate, pH 3 and acetonitrile, respectively. The initial concentration of mobile phase B was 10% and this condition was maintained for 0.2 min, followed by a linear increase of B to 95% over 0.2 min, held for 1 min. The initial concentration was then reinstated and held for 1.2 min for re-equilibration.

Accuracy of standards and inter-assay variability were within ± 15%. Detailed MS conditions were shown in Table 1.

**Sigmoidal dose-response to calculate EC50.** The apparent EC50 (Fig. 3C) of the elacridar at half maximal increase of \( K_{p,\text{brain}} \) of digoxin was calculated in GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego, USA) using the following equation:

\[
Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{\left(\text{EC50} - X\right) / \text{Hillslope}}}
\]

Bottom: \( K_{p,\text{brain}} \) of digoxin in the absence of elacridar
Top: $K_{p,brain}$ of digoxin at the maximum plateau in the presence of elacridar

Hillslope: steepness of the sigmoidal dose-response curve

**Statistics.** Statistical significance of difference between mean values was tested using a two-tailed unpaired Student’s t test and one-way analysis of variance followed by Dunnet’s comparison test. Differences with a $p$-value of less than 0.05 were considered to be statistically significant.
Results

Inhibition of rat Mdr1a-mediated digoxin transport by P-gp inhibitors. To investigate the inhibitory potency of P-gp inhibitors (elacridar, itraconazole, cyclosporin A, ketoconazole, verapamil, and quinidine), we performed a transcellular transport study in rat Mdr1a-expressing LLC-PK1 cells using digoxin as a probe substrate of P-gp. *In vitro* $K_i$ values for digoxin transport via rat Mdr1a were calculated according to the intersections of Dixon plots (Fig. 2). Elacridar showed the most potent P-gp inhibitory effect among six P-gp inhibitors tested (Table 2).

Drug interaction study of digoxin and elacridar in rats. To elucidate the effect of P-gp inhibition on the digoxin transport to the brain, intravenous infusion of digoxin in the presence or absence of elacridar was performed in rats. During the infusion of digoxin and elacridar, the plasma concentration reached steady-state (Fig. 3A and B). The brain-to-plasma concentration ratio ($K_{p,brain}$) of digoxin was measured at 8 h after the initiation of administration. Elacridar notably increased $K_{p,brain}$ of digoxin by approximately 14-fold relative to the control. The apparent EC$_{50}$ of elacridar was approximately 266 ng/mL (Fig. 3C). Elacridar plasma concentration increased via elevation of infused drugs, without significant change in the plasma concentration of digoxin ($p > 0.05$). In the presence of elacridar, there is no significant change in the unbound fraction in plasma ($f_p$) or in brain ($f_b$) (data not shown).

Drug interaction study of digoxin and clinically used P-gp inhibitors in rats. To investigate whether clinically used P-gp inhibitors cause DDI on the digoxin transport to the brain, intravenous infusion of digoxin in the presence or absence of itraconazole, cyclosporin A, ketoconazole, verapamil and quinidine was performed. The plasma concentration of each P-gp inhibitor reached a pseudo steady-state condition within 16 h infusion. The $K_{p,brain}$ of digoxin in the presence of cyclosporin A increased in a dose-dependent manner and had a significantly higher (0.33±0.07) values than that of control values (0.20±0.04) at the highest doses (10 mg/h/kg) ($p < 0.05$). On the
contrary, the other compounds evaluated did not significantly increase $K_{p,\text{brain}}$ of digoxin in rats ($p > 0.05$) (Fig. 4).

**IVIVC of P-gp-mediated DDI in rats.** The predictability of P-gp-mediated DDI at the rat BBB was evaluated using the IVIV correlation. Given that unbound plasma concentration of P-gp inhibitors ($[I_{\text{unbound}}]$) is the effective concentration for P-gp inhibition, the relationship between *in vivo* $K_{p,\text{brain}}$ of digoxin and $[I_{\text{unbound}}]/K_i$ was investigated. The IVIVC plots showed a positive correlation and indicated that the compounds showing $[I_{\text{unbound}}]/K_i > 1$ increased $K_{p,\text{brain}}$ of digoxin in rats. On the other hand, the data points corresponding to the compounds with $[I_{\text{unbound}}]/K_i < 1$ mostly lay on the dashed horizontal line, which showed the control $K_{p,\text{brain}}$ of digoxin (Fig. 5).
Discussion

P-gp is a well-studied ABC transporter, and potential P-gp-mediated DDI studies are currently focused on drug candidates especially acting on the central nerve system (Tsuji, 2006; Feng et al., 2008). However, little quantitative information is available regarding potential P-gp-mediated DDIs at the BBB. We have therefore quantitatively investigated the impact of P-gp inhibition through the use of in vitro-in vivo correlation at the rat BBB.

In vitro P-gp inhibition studies have been performed in pharmaceutical industries and academic fields to predict in vivo DDI of digoxin. However, the evaluation method of in vitro P-gp inhibitory activity has been still controversial (Adachi et al., 2001; Keogh and Kunta, 2006; Rautio et al., 2006). A major challenge of in vitro P-gp inhibition study is to theoretically and accurately determine the intrinsic efflux activity (PSP-gp in Fig. 1). An additional challenge is to correct the influence of the different passive permeability between rat Mdr1a and mock cells. The third challenge is to ascertain in vitro Ki values that are independent of the affinity and concentration of probe substrates. To overcome these issues, we employed a theoretical approach to ascertain in vitro Ki values using the parameter MCFR. According to the compartment model shown in Fig.1, MCFR across rat Mdr1a-expressing cells / rat Mdr1a-inhibited cells can be denoted as 1+PS P-gp/PSA,eff (see equation 4 in Material and Methods section). Assuming that the permeability coefficient for the efflux transport across the apical membrane (PSA,eff) is not affected by P-gp inhibitors, [MCFR-1] indicates the intrinsic efflux activity. In vitro Ki values were estimated using a linearized version of the Michaelis-Menten equation (Dixon plot, Fig. 2). The rank order of Ki value was in good agreement with previous reports focused on P-gp inhibition using MDR1-MDCKII (Rautio et al., 2006). Hence, we have successfully determined in vitro Ki values that theoretically reflect the intrinsic P-gp efflux activity.

In vivo Kp,brain of digoxin was determined in the presence or absence of P-gp inhibitors with a constant intravenous infusion in rats. Under the steady-state condition, Kp,brain can be interpreted as the ratio of uptake clearance from the systemic blood to brain to efflux clearance from the brain to systemic blood. This definition implies that the process of digoxin transport into brain may not only
mediate the efflux process, but also the influx process. P-gp and BCRP (breast cancer resistance protein) are representative transporters of the efflux process and OATP (organic anion transport peptides) is a representative transporter of the influx process. In terms of the efflux transporter, P-gp plays a significant role in transporting digoxin in vitro and in vivo, but not BCRP (Pavek et al., 2005). For the influx transporter, one subtype of the OATP family (Oatp1a4) may play a role in the transport of digoxin as well as P-gp in rats. Oatp1a4 is expressing in both the luminal and anti-luminal membrane of the BBB and plays a role in the uptake of organic anionic compounds (i.e. estradiol-17β-glucuronide, statins, and taurocholate) (Nee et al., 1997; Gao et al., 1999). However, a recent study using Oatp1a4(-/-) mice revealed that the blood-to-brain uptake of digoxin was not significantly different between Oatp1a4(-/-) and wild-type mice unless Oatp1a4(-/-) mice were pretreated with elacridar (Ose et al., 2010). Although a species difference of the function of Oatp1a4 between rats and mice is arguable, OATP may have a much less impact than P-gp on the digoxin transport to the rat brain. Hence, the evaluation of $K_{p,\text{brain}}$ of digoxin in the presence or absence of P-gp inhibitors is a rational approach to understand the impact of P-gp-mediated DDI at the rat BBB.

The magnitude of P-gp inhibition at the BBB was evaluated in rats. In the presence of elacridar in the rat plasma, $K_{p,\text{brain}}$ of digoxin was increased by approximately 14-fold over the control (Fig. 3C). This result was comparable to a previous report in which $K_{p,\text{brain}}$ of digoxin in Mdr1a/1b(-/-) mice was about 19-fold higher than that in wild-type mice (Schinkel et al., 1994). Additionally, the apparent EC$_{50}$ of elacridar plasma concentration to achieve chemically induced P-gp knockout in rats was 266 ng/mL. This result was also comparable to the previous report (EC$_{50}$$\approx$320 ng/mL) using SB-487946 as P-gp probe substrate (Cutler et al., 2006). The unbound EC$_{50}$ was approximately 2.7 ng/mL (4.7 nmol/L), which was in good agreement with the in vitro $K_{i}$ value (Table 2). These results indicated that elacridar was the useful compound to investigate the maximum effect by P-gp inhibition at the rat BBB. In order to estimate the effect of clinically used P-gp inhibitors on the digoxin transport, itraconazole, cyclosporin A, ketoconazole, verapamil, and quinidine were selected, because these drugs have been concomitantly administered with digoxin in clinical trials.
(Angelin et al., 1987; Dorian et al., 1988; Hedman et al., 1991; Jalava et al., 1997b; Larsen et al., 2007). The $K_{\text{p,brain}}$ of digoxin significantly increased in the presence of cyclosporin A, but not other P-gp inhibitors. This experimental result can be explained by the fact that cyclosporin A showed a relatively strong P-gp inhibitory effect ($K_i$: 0.038 μmol/L, Table 2) among P-gp inhibitors and has sufficient exposure in rat plasma (~3000 ng/mL). Consequently, P-gp-mediated DDI of digoxin at the rat BBB may be observed under sufficient exposure of potent P-gp inhibitors in rat plasma.

In order to quantify P-gp-mediated DDIs at the rat BBB, we employed the IVIV approach. According to the IVIVC plot, in vivo $K_{\text{p,brain}}$ of digoxin and $\left[I,\text{unbound}/K_i\right]$ showed a positive correlation (Fig. 5). Compounds showing $\left[I,\text{unbound}/K_i\right] > 1$ increased $K_{\text{p,brain}}$ of digoxin in rats. For example, the values of $\left[I,\text{unbound}/K_i\right]$ for elacridar and cyclosporin A, which demonstrated a significant effect on $K_{\text{p,brain}}$ of digoxin, ranged from 0.57 to 5.34 and 0.33 to 1.56, respectively. The assessment of drug distribution in the central nerve system should be evaluated with unbound plasma concentrations, because tissue partition coefficients such as $K_{\text{p,brain}}$ may be influenced by the relative binding affinity of a substrate for proteins in plasma (Kalvass and Pollack, 2007). Our investigation provided the theoretically reasonable cutoff limit ($[I,\text{unbound}/K_i]$ of 1) for the risk of P-gp-mediated DDI at the rat BBB.

Based on the assumption that there is little species difference in both intrinsic activity and expression level of P-gp between rats and human, we examined the possibility of P-gp-mediated DDI at the human BBB taking in vitro $K_i$ values in human MDR1 and plasma concentration at the conventional therapeutic dose into account. In vitro $K_i$ values were determined using MCFR in human MDR1-expressing LLC-PK1 cells and were summarized in Table 3. Although the $K_i$ value of cyclosporin A in human MDR1 was more than 10 times higher than that in rat Mdr1, notable species difference of in vitro $K_i$ values was not observed in other compounds. Estimated values of $[I,\text{unbound}/K_i]$ for itraconazole, cyclosporin A, ketoconazole, verapamil, and quinidine in humans were less than 1, where $[I,\text{unbound}]$ is the normal therapeutic unbound plasma concentration of P-gp inhibitors in the clinical use (Brunton et al., 2005). Among the investigated P-gp inhibitors, quinidine showed the highest $[I,\text{unbound}/K_i]$ value. In fact, the opiate-induced respiratory depression of
loperamide was reported with a concomitant administration of quinidine at a dose of 600 mg (Sadeque et al., 2000), relatively higher than the conventional clinical dosage (Brunton et al., 2005). This result can be explained by the fact that quinidine likely causes P-gp inhibition at a high dosage. It is also notable that the plasma protein binding of quinidine is relatively lower than other P-gp inhibitors. Based on the IVIVC relationship, we assume that P-gp-mediated DDI at the human BBB may be limited because no compound showed \( \frac{I_{unbound}}{K_i} > 1 \) at clinical doses (Table 4).

To date, neither the species difference of expression level of mdr1a / MDR1 nor the anatomical structure in the cerebral endothelial cells, which may affect the accurate understandings of P-gp related DDI at the BBB, has been clarified. Further studies are required to address these issues.

In summary, this is the first quantitative IVIVC investigation to evaluate the impact of P-gp inhibition on the digoxin transport across the rat BBB. The degree of \textit{in vitro} P-gp inhibition was evaluated by \( \text{Ki} \) values using MCFR, which reflected the intrinsic P-gp efflux activity. The effect of \textit{in vivo} P-gp inhibition was evaluated in terms of the increase in \( K_{p,brain} \) of digoxin in the presence of P-gp inhibitors. The obtained IVIVC plots indicated that P-gp-mediated DDI occurred in the presence of compounds with \( \frac{I_{unbound}}{K_i} > 1 \) in rats. On the basis of the IVIVC in rats, we speculate that clinically used P-gp inhibitor may not cause P-gp inhibition at the human BBB in light of \textit{in vitro} \( K_i \) values in human MDR1 and plasma concentrations at conventional therapeutic doses.
Authorship Contributions

Participated in research design: Sugimoto, Hirabayashi, Amano, and Moriwaki

Conducted experiments: Sugimoto, Hirabayashi, Kimura, and Furuta

Contributed new reagents or analytic tools: not applicable

Performed data analysis: Sugimoto

Wrote or contributed to the writing of the manuscript: Sugimoto, Hirabayashi, Amano, and Moriwaki

Other: not applicable.
References


Legends for Figures

Fig. 1. Schematic diagram of MDR1-expressing or MDR1-inhibited cells. P-gp expresses on the apical membrane and transports substrates from intracellular area into the apical chamber of transwell. Passive permeability at the apical and basal membrane consists of the influx and efflux process. MDR1-inhibited cells was designed by adding 4 μmol/L elacridar in both the apical and basal chambers.

Fig. 2. Dixon plots showing the inhibitory effect of elacridar (A), itraconazole (B), cyclosporin A (C), ketoconazole (D), verapamil (E), and quinidine (F) on MCFR of digoxin by rat Mdr1a-expressing LLC-PK1 cells. MCFR is defined as the flux ratio of digoxin (open circle, 10; open triangle, 20; closed circle, 50; closed triangle, 250 μmol/L) in rat Mdr1a-expressing LLC-PK1 cells divided by the corresponding ratio in functionally MDR1-inhibited cells using 4 μmol/L of elacridar in the presence of each inhibitor (A: elacridar, 0.0024, 0.08, 0.24 μmol/L, C: cyclosporin A, 0.03, 0.1, 0.3 μmol/L, D: ketoconazole, 1, 3, 10 μmol/L, B, E, F: itraconazole, verapamil, and quinidine: 0.3, 1, 3 μmol/L). The transport study was conducted at 37ºC for 1h. Each result represents the mean value (n = 3).

Fig. 3. Profiles of time versus plasma concentrations of digoxin (A) and elacridar (B) during intravenous infusion and plasma concentration of elacridar versus $K_{p,\text{brain}}$ of digoxin in rats (C). Digoxin (0.06 mg/h/kg) and elacridar (open square: 0.125, open diamond: 0.25, closed circle: 0.5, closed square: 1, closed triangle: 2 mg/h/kg) were co-infused intravenously for 8 h in rats. Plasma concentrations of digoxin (A) and elacridar (B) were determined by LC/MS/MS. $K_{p,\text{brain}}$ of digoxin following 8 h continuous infusion was shown in (C). The apparent EC$_{50}$ was defined as the elacridar concentration at half maximal increase of $K_{p,\text{brain}}$ of digoxin. The plasma concentration of digoxin was not significantly different between in the presence or absence of elacridar at a level of $p > 0.05$. The values were expressed as mean ± S.D. (n = 4).
Fig. 4. The $K_{p,\text{brain}}$ of digoxin following 16 h continuous infusion of digoxin in the presence of itraconazole, cyclosporin A, ketoconazole, verapamil, and quinidine in rats. Digoxin (0.06 mg/h/kg) and itraconazole (0.01, 0.03 mg/h/kg), cyclosporin A, verapamil (1, 3, 10 mg/h/kg), ketoconazole, or quinidine (1, 3, 10 mg/h/kg) were co-infused intravenously for 16 h in rats. * indicated $K_{p,\text{brain}}$ of digoxin was significantly different from that of the control ($p < 0.05$). The values were expressed as mean ± S.D. (n = 4).

Fig. 5. Relationship between in vivo $K_{p,\text{brain}}$ of digoxin and $[I_{\text{unbound}}] / K_i$ in rats. The $K_{p,\text{brain}}$ of digoxin in the presence of elacridar (open circle), itraconazole (open triangle), cyclosporin A (open square), ketoconazole (closed circle), verapamil (closed square), quinidine (closed triangle) and in the absence of P-gp inhibitors (control, dashed line) versus $[I_{\text{unbound}}] / K_i$. The $[I_{\text{unbound}}]$ indicated unbound plasma concentrations of inhibitor at pseudo steady-state condition.
## Table 1. Analytical condition in LC/MS/MS analysis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ionization mode</th>
<th>Detection mode</th>
<th>m / z</th>
<th>Source temperature (°C)</th>
<th>Mass spectrometric parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digoxin</td>
<td>Electrospray ionization</td>
<td>Multiple reaction monitoring (MRM)</td>
<td>825.5 / 779.4</td>
<td>450</td>
<td>CE: -32, DP: -90, EP: -10, CXP: -15</td>
</tr>
<tr>
<td>Elacridar</td>
<td></td>
<td></td>
<td>564.2 / 252.2</td>
<td>550</td>
<td>CE: 55, DP: 100, EP: 10, CXP: 15</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>Electrospray ionization</td>
<td></td>
<td>705.4 / 450.4</td>
<td>550</td>
<td>CE: 40, DP: 100, EP: 10, CXP: 15</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>Electrospray ionization</td>
<td></td>
<td>1202.8 / 100.3</td>
<td>550</td>
<td>CE: 80, DP: 108, EP: 10, CXP: 8</td>
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<tr>
<td>Ketoconazole</td>
<td></td>
<td></td>
<td>531.3 / 489.3</td>
<td>550</td>
<td>CE: 30, DP: 100, EP: 10, CXP: 15</td>
</tr>
<tr>
<td>Quinidine</td>
<td></td>
<td></td>
<td>326.3 / 308.1</td>
<td>550</td>
<td>CE: 33, DP: 61, EP: 10, CXP: 26</td>
</tr>
</tbody>
</table>

I.S.: internal standard
Table 2. *In vitro* \( K_i \) values for digoxin transport via rat Mdr1a

<table>
<thead>
<tr>
<th>Compound</th>
<th>( K_i ) (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elacridar</td>
<td>0.0016</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.67</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>0.038</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>1.41</td>
</tr>
<tr>
<td>Verapamil</td>
<td>0.95</td>
</tr>
<tr>
<td>Quinidine</td>
<td>2.24</td>
</tr>
<tr>
<td>Mean (n = 3)</td>
<td></td>
</tr>
</tbody>
</table>
### Table 3. *In vitro* Ki values for digoxin transport via human MDR1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ki (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elacridar</td>
<td>0.0025</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.57</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>0.45</td>
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<tr>
<td>Ketoconazole</td>
<td>1.78</td>
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<tr>
<td>Verapamil</td>
<td>0.98</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.93</td>
</tr>
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</table>

Mean (n = 4)
<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/man)</th>
<th>Peak plasma concentration (μmol/L)</th>
<th>Unbound fraction in human plasma</th>
<th>$[\text{I,unbound}] / K_i$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Itraconazole</td>
<td>200</td>
<td>0.92</td>
<td>0.0020 ± 0.0007</td>
<td>&lt; 0.01 (Jalava et al., 1997a)</td>
<td></td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>840</td>
<td>0.92 - 1.11</td>
<td>0.045 ± 0.011</td>
<td>0.09 - 0.11 (Brunton et al., 2005)</td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>200</td>
<td>1.40 - 4.50</td>
<td>0.0071 ± 0.0017</td>
<td>0.01 - 0.02 (Badcock et al., 1987)</td>
<td></td>
</tr>
<tr>
<td>Verapamil</td>
<td>240</td>
<td>0.26 - 0.36</td>
<td>0.093 ± 0.014</td>
<td>0.02 - 0.03 (McTavish and Sorkin, 1989)</td>
<td></td>
</tr>
<tr>
<td>Quinidine</td>
<td>400</td>
<td>1.67 - 4.81</td>
<td>0.16 ± 0.03</td>
<td>0.29 - 0.85 (Ueda et al., 1976)</td>
<td></td>
</tr>
</tbody>
</table>

$[\text{I,unbound}]$: normal therapeutic unbound plasma concentrations in humans

Unbound fraction in human plasma (mean ± S.D. n = 4-5)
Figure 1

MDR1 cells

MDR1-inhibited cells
Figure 2

A. Elacridar

B. Itraconazole

C. Cyclosporin A

D. Ketoconazole

E. Verapamil

F. Quinidine
Figure 3

A

B

C

Digoxin concentration in plasma (ng/mL)

Elacridar concentration in plasma (ng/mL)

$K_{p,brain}$ of digoxin

Time after administration (h)

Time after administration (h)

Plasma concentration of elacridar (ng/mL)

% Control

Elacridar (0.125 mg/h/kg)

Elacridar (0.25 mg/h/kg)

Elacridar (0.5 mg/h/kg)

Elacridar (1 mg/h/kg)

Elacridar (2 mg/h/kg)
Figure 4

![Bar graph showing the effect of various drugs on the brain concentration of digoxin.](image)