

Recognition Mechanism of Dibenzoylhydrazines
by Human P-glycoprotein

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Abbreviations

AP	apical
BBB	blood-brain barrier
BCRP	breast cancer resistance proteins
BL	basolateral
r^2	coefficient of determination
CNS	central nervous system
CoMFA	comparative molecular field analysis
DBHs	dibenzoylhydrazines
DDI	drug-drug interactions
EMA	European Medicines Agency
ESI	electrospray ionization
esp	electrostatic potential
FBS	fetal bovine serum
FDA	Food and Drug Administration
GA	Genetic Algorithm
HBSS	Hank's balanced salt solution
IS	internal standard
$K_{p,brain}$ value	ratio of brain concentration/plasma concentration
LLC-GA5-COL150 cells	LLC-PK1 cells transfected with human <i>MDR1</i>
LLC-PK1 cells	porcine kidney epithelial cells
MDR	multidrug resistance
MRM	multiple reaction monitoring
MRP	multidrug resistance proteins
NBD	nucleotide-binding domain
P_{app}	apparent permeability coefficient
PBS	phosphate-buffered saline
PET	positron emission tomography
P-gp	P-glycoprotein
QSAR	quantitative structure-activity relationship
SNPs	single nucleotide polymorphisms
TEER	transepithelial electrical resistance
TMD	transmembrane domain

General Introduction

Human and animals have evolved metabolic and efflux systems to protect themselves from xenobiotics, including natural products, industrial chemicals, and agrochemicals. P-glycoprotein (P-gp, also known as MDR1 or ABCB1), which is encoded by the *MDR1* gene, is a member of the ATP-binding cassette transporter family. Human P-gp is a 170-kDa plasma membrane protein containing 1,280 amino acids and consists of 2 homologous halves, each of which contains 6 transmembrane α -helices (transmembrane domain, TMD) and a nucleotide-binding domain (NBD) (Chen *et al.*, 1986; Kartner *et al.*, 1983). Schematic diagrams of P-gp structure and transport mechanism by P-gp were shown in Figure 1. P-gp transports a wide variety of drugs and xenobiotics out of cells and functions as an ATP-dependent efflux pump (Figure 2). P-gp is strongly expressed in a number of organs such as the luminal membrane of the small intestine, colon, blood-brain barrier (BBB), and biliary canalicular membranes of hepatocytes (Thiebaut *et al.*, 1987), and plays a significant role in drug absorption and disposition.

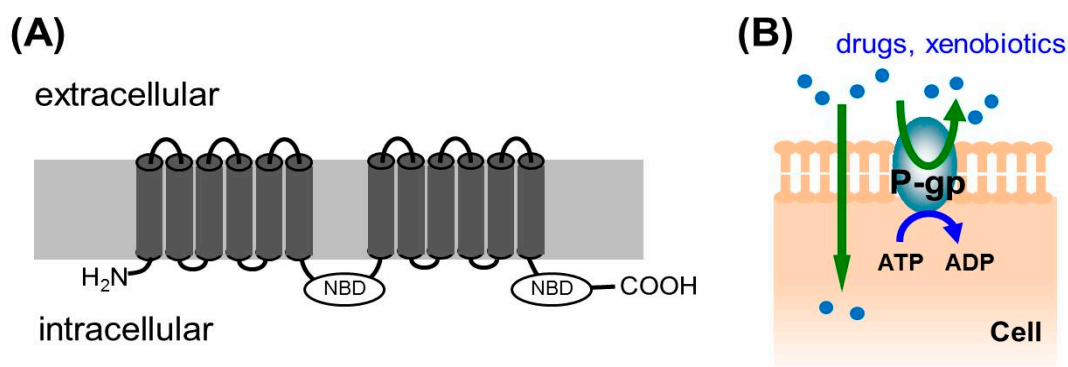


Figure 1. (A) Schematic diagrams of P-gp structure (B) Transport mechanism of drugs and xenobiotics by P-gp.

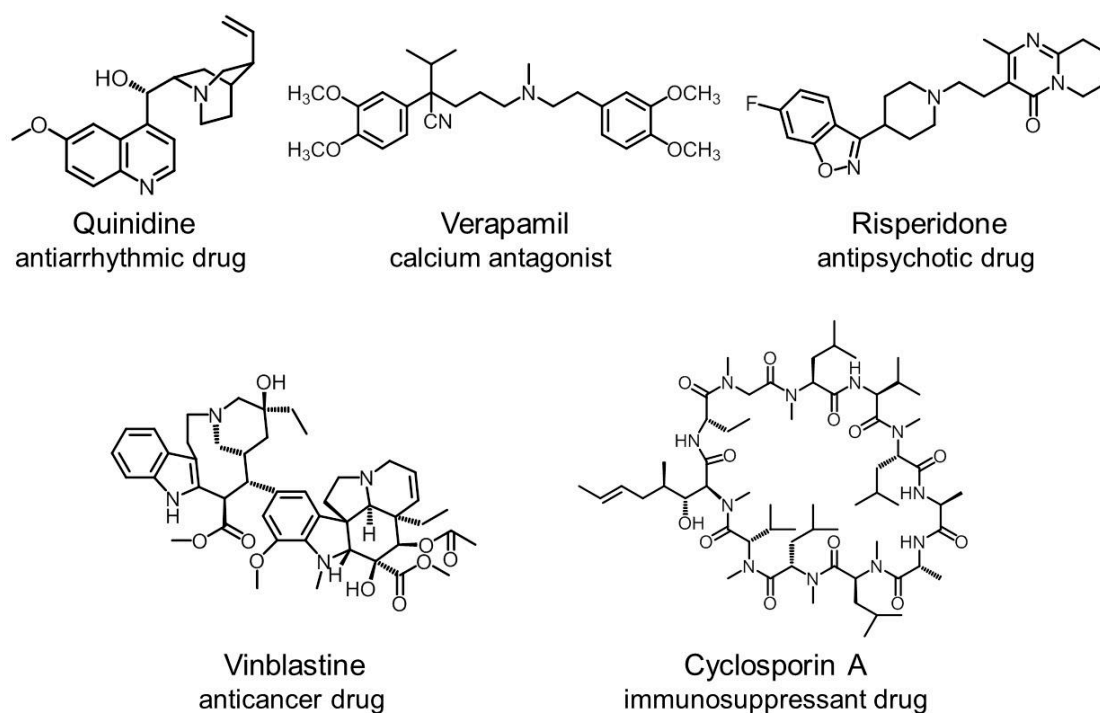


Figure 2. Structure of P-gp substrates.

In the intestines, P-gp limits the oral absorption of drugs from the gastrointestinal tract into the systemic circulation. In the liver, it is involved in the biliary excretion of drugs. BBB is composed of capillary endothelial cells in which tight junctions are highly developed (Abbott *et al.*, 2010), and expresses various efflux transporters such as

P-gp, multidrug resistance proteins (MRP), and breast cancer resistant proteins (BCRP) (Schinkel., 1999; Ohtsuki and Terasaki, 2007). These efflux transporters and tight junctions limit the penetration of drugs and xenobiotics into the brain and protect the central nervous system (CNS). Of these, P-gp is the most studied and well-understood transporter with regards to brain drug disposition, with many studies showing that P-gp restricts the brain penetration of various drugs and xenobiotics. Previous studies using the *mdr1a/1b* gene knockout mouse have shown that the ratio of the brain concentration/plasma concentrations ($K_{p,brain}$ values) of several drugs such as verapamil, digoxin, quinidine, and ivermectin (P-gp substrates) were significantly higher in knockout mice than in wild-type mice (Doran *et al.*, 2005; Schinkel *et al.*, 1994; Schinkel *et al.*, 1995; Liu *et al.*, 2008). Furthermore, the brain concentration of ivermectin, an anti-parasitic drug for animals and humans, was found to be 100-fold higher in *mdr1a* gene knockout mice than in wild-type mice, and *mdr1a* gene knockout mice administered ivermectin died due to neurotoxicity (Schinkel *et al.*, 1994). Human positron emission tomography (PET) studies (Sasongko *et al.*, 2005) also showed that the $K_{p,brain}$ value of ^{11}C -radiolabeled verapamil was 1.88-fold higher in the presence of cyclosporine A (P-gp inhibitor). These findings indicate that P-gp significantly affects the efficacies and/or toxicities of drugs. Thus, P-gp-mediated drug-drug interactions (DDI) may occur in various organs and tissues (Lija *et al.*, 2003; Sasongko *et al.*, 2005), and evaluations of the potential for DDI are considered critical for drug development (FDA Guidance, 2012; EMA Guideline, 2012).

P-gp also recognizes various anticancer drugs such as vinca alkaloids (vinblastine and vincristine), taxanes (paclitaxel and docetaxel), and anthracyclines (doxorubicin and daunorubicin) (Schinkel and Jonker, 2003). Since decreases in the uptake of anticancer

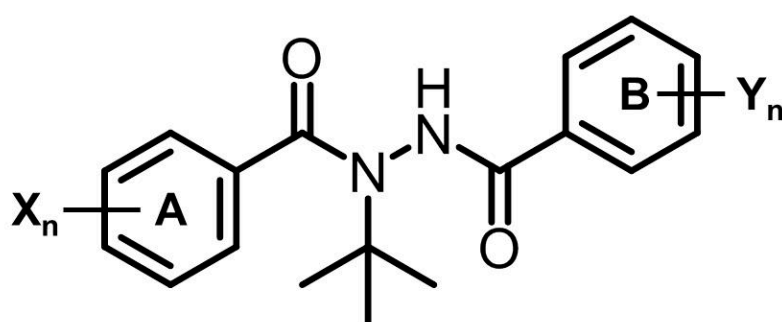
drugs into cancer cells by P-gp reduce the efficacy of chemotherapy, P-gp plays a significant role in multidrug resistance (MDR) in the treatment of cancers (Ueda *et al.*, 1984; Klukovits and Krajcsi, 2015). Thus, the design of drugs that will not interact with P-gp is important for the development of drugs that target the CNS, and anticancer drugs. Conversely, P-gp modulators have been shown to inhibit P-gp function and increase the uptake of other compounds into cells (Klukovits and Krajcsi, 2015; Pajeva and Wiese, 2009). P-gp modulators are expected to increase the efficacy of anticancer drugs when co-administered with them.

A number of single nucleotide polymorphisms (SNPs) have been detected in the human *MDR1* gene (Wolking *et al.*, 2015). Previous studies demonstrated that SNPs in the *MDR1* gene affected the pharmacokinetics of drugs (Hoffmeyer *et al.*, 2000; Kurata *et al.*, 2002); however, contradictory findings have also been reported (Choway *et al.*, 2005; Gerloff *et al.*, 2002). SNPs have been identified in dogs (Mealey *et al.*, 2001; Pulliam *et al.*, 1985). Mealey *et al.* (2001) detected an SNP in the *Mdr1* gene in a subpopulation of Collie dogs (ivermectin-sensitive Collies). These ivermectin-sensitive Collies showed increased neurotoxicity to ivermectin due to the markedly increased brain concentration of ivermectin (Pulliam *et al.*, 1985).

Diverse agrochemicals have been evaluated for their interactions with human P-gp, and several compounds have been shown to inhibit P-gp-mediated transport in intact cells (Bain and LeBlanc, 1996) and membrane vesicles (Mazur *et al.*, 2015). Sreeramulu *et al.* (2007) also revealed that several commonly used insecticides induced P-gp ATPase activity using mammalian P-gp reconstituted in liposomes. Agrochemicals have improved farm work efficiency and crop production, and contributed to the stable supply of agricultural products. However, the impact of agrochemicals on non-target

organisms including humans needs to be considered. The P-gp-mediated transport processes of agrochemicals may influence pharmacokinetic interactions with other chemicals including drugs. Thus, determining whether agrochemicals are substrates and/or inhibitors of P-gp is useful for their risk assessments even if the agrochemicals themselves are not toxic.

In a previous report (Kanaoka *et al.*, 2013), diverse chemicals including agrochemicals were screened by measuring ATPase activity with human P-gp reconstituted in membrane vesicles in order to investigate the interactions between agrochemicals and human P-gp. In the report, it was found that dibenzoylhydrazines (DBHs) such as tebufenozide and methoxyfenozide (Figure 3), which are insect growth regulators that bind to the ecdysone receptor of insects (Billas *et al.*, 2003), stimulated ATPase activity. However, it is unknown whether these DBHs are actually transported by P-gp.



Tebufenozide: X_n = 3,5-dimethyl, Y_n = 4-ethyl

Methoxyfenozide: X_n = 3,5-dimethyl, Y_n = 2-methyl, 3-methoxy

Figure 3. Structures of dibenzoylhydrazines.

In this study, the author selected DBHs with structurally diverse substituents on the B ring as model compounds in order to elucidate the mechanisms of substrate recognition by P-gp. In Chapter 1, it was determined whether DBHs are actually transported by P-gp using both the *in vitro* bidirectional transport assay and the *in vivo* study of rats. The inhibitory effects of DBHs on quinidine (a P-gp substrate) transport by P-gp were also examined in order to ascertain whether these derivatives are inhibitors of P-gp. In Chapter 2, the author evaluated the inhibition of P-gp-mediated quinidine transport by two series of DBHs (18 compounds having 3,5-Me₂ group and 40 compounds having 2-Cl group on the A-ring) and performed classical quantitative structure-activity relationships (QSAR) and 3D-QSAR analyses using inhibitory activities as the indicator of interaction strength between DBHs and P-gp. In Chapter 3, the docking simulation for two series of DBHs was conducted in order to propose their binding sites and modes.

CHAPTER 1:

***In vitro* and *in vivo* evaluations of the human P-glycoprotein-mediated efflux of dibenzoylhydrazines**

1-1. Introduction

Several *in vitro* and *in vivo* methods have been used to identify P-gp substrates. *In vitro* methods include ATPase activity, uptake inhibition, and cell-based bidirectional transport assays (Table 1-1) (Polli *et al.*, 2001; Feng *et al.*, 2008; Zhang *et al.*, 2003). The amount of ADP or inorganic phosphate generated by ATP hydrolysis is monitored in the ATPase activity assay using P-gp reconstituted in membrane vesicles. It is associated with the binding of substrates to P-gp followed by structural conversion. The inhibitory effects of test compounds on the uptake of probe fluorescent substrates such as Calcein-AM or Rhodamine123 are evaluated in the uptake inhibition assay using intact cells or membrane vesicles. The transport of substrates is measured in the cell-based bidirectional transport assay using polarized epithelial cells cultured on transwell plates. ATPase activity and uptake inhibition assays are suitable for high-throughput screening; however, difficulties are associated with determining whether the test compound is directly transported by P-gp using these assay methods (Polli *et al.*, 2001; Zhang *et al.*, 2003). On the other hand, unlike other *in vitro* methods, it is possible to directly measure the transport of compounds in the bidirectional transport assay; however, this assay is time consuming. In animal studies, comparisons

of the brain distribution of test compounds in wild-type mice or rats with and without P-gp inhibitors (Sugimoto *et al.*, 2011; Loureiro *et al.*, 2015; Song *et al.*, 1999), or in P-gp knockout and wild-type mice (Doran *et al.*, 2005; Schinkel *et al.*, 1994; Schinkel *et al.*, 1995; Liu *et al.*, 2008) are widely used.

As described in General Introduction, DBHs such as tebufenozide and methoxyfenozide stimulated ATPase activities (Kanaoka *et al.*, 2013); however, it currently remains unclear whether these derivatives are actually transported by P-gp and inhibit transport of other chemicals by P-gp. Although DBHs are safe pesticides based on their mode of action (LD₅₀ value of tebufenozide and methoxyfenozide for rats and mice: > 5,000 mg/kg) (Tomlin, 2009), it is important to establish whether they are substrates and/or inhibitors of P-gp based on their pharmacokinetic interactions with other chemicals as a part of risk assessments of agrochemicals.

Table 1-1. Summary of *in vitro* methods to identify P-gp substrates.

Assay Type	Method	Characteristics
ATPase activity	ATPase activity is evaluated using P-gp reconstituted in membrane vesicles	• Suitable for high-throughput screening • Cannot determine whether the compound is directly transported by P-gp
Uptake inhibition	Uptake inhibition of probe fluorescent substrate is evaluated using intact cells or membrane vesicle	
Bidirectional transport	Transport of substrates is measured directly using polarized epithelial cells cultured on transwell plates	• Can measure the transport directly • Can distinguish substrates from inhibitors • Time consuming • Strong correlation of <i>in vivo</i> P-gp function • Cannot identify substrates with high passive permeability

In this chapter, in order to evaluate the interactions of DBHs with other chemicals in humans, it was determined whether DBHs are P-gp transport substrates using both the *in vitro* bidirectional transport assay with MDR1-LLC-PK1 cells, which overexpress P-gp, and the *in vivo* study of rats. In the *in vivo* study, the influence of P-gp inhibitors (elacridar and valspodar, Figure 1-1) on the brain to plasma ratio of methoxyfenozide (relatively higher aqueous soluble compound among DBHs) was investigated in rats. Based on the result, the usefulness of assay methods for identification of P-gp substrates was discussed. The inhibitory effects of DBHs on quinidine (a P-gp substrate) transport by P-gp was also examined in order to ascertain whether these derivatives are inhibitors of P-gp and influence the absorption and disposition of other chemicals. Finally, the risk of DBHs caused by interaction with other chemicals including drugs was evaluated by considering the DBHs' potential as the substrates and inhibitors of P-gp as well as their plasma concentrations.

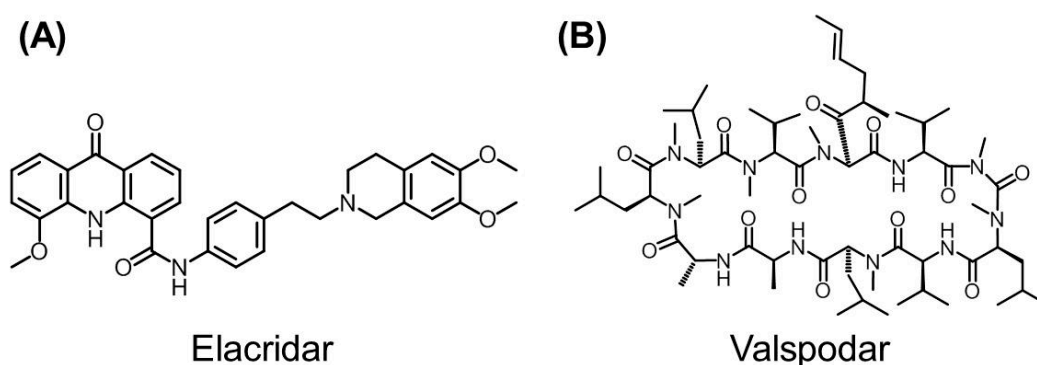


Figure 1-1. Structures of elacridar (A) and valspodar (B).

1-2. Materials and Methods

1-2-1. Chemicals

Tebufenozide, methoxyfenozide, and elacridar were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Valspodar and quinidine were obtained from Sigma-Aldrich (MO, USA). Fetal bovine serum (FBS) was purchased from the American Type Culture Collection (ATCC, MD, USA). All other chemicals were of analytical grade, and purchased either from Sigma-Aldrich (MO, USA) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

1-2-2. Animals

Male Crl:CD (SD) rats were purchased from Japan SLC Inc. (Shizuoka, Japan), and used in experiments at 7-10 weeks old with a body weight of 222-342 g. Rats were maintained on a 12-h light/dark cycle in a temperature-controlled environment. Animals had free access to food and water. Animal experiments were performed according to the guidance provided by Otsuka Pharmaceutical Co., Ltd., Guidelines for Animal Care and Use.

1-2-3. Cell culture

LLC-PK1 cells (porcine kidney epithelial cells) were obtained from ATCC (MD, USA). LLC-GA5-COL150 cells (LLC-PK1 cells transfected with human *MDR1*) (Tanigawara *et al.*, 1992; Ueda *et al.*, 1992) were obtained from the RIKEN BioResource Center (Ibaraki, Japan). LLC-PK1 cells were grown in Medium 199 containing 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO₂. Colchicine (150 ng/mL) was added to the medium for LLC-GA5-COL150 cells. LLC-PK1 cells and LLC-GA5-COL150 cells were used to prepare cell monolayers after thawing and subculturing every 7 days.

1-2-4. Bidirectional transport assay across LLC-PK1 and LLC-GA5-COL150 cells

LLC-PK1 cells and LLC-GA5-COL150 cells were seeded on a 24-well Transwell (Millicell 24, PCF 0.4 μm pore size, Merck Millipore Ltd., MA, USA) at a density of 3.6×10^5 cells/cm². LLC-PK1 cells were grown in Medium 199 containing 10% FBS, 100 units/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin at 37°C in 5% CO₂. LLC-GA5-COL150 cells were grown in the presence of colchicine (150 ng/mL). Medium was replaced with fresh medium on the third and seventh days after seeding. Colchicine-free medium was used on the seventh day. Cells were used in the transport study 8-10 days after seeding. Transepithelial electrical resistance (TEER) was measured in the well before use, and TEER in all wells was 150 $\Omega \times \text{cm}^2$ or more. Apical (AP) to basolateral (BL) and BL to AP transport was evaluated (Figure 1-2). Cells were washed 2 times with transport buffer (Hank's balanced salt solution (HBSS) containing 25 mM D-Glucose and 10 mM HEPES, pH 7.4), and preincubated for 30 min in transport buffer at 37°C. The volumes of the AP and BL sides were 400 and 800 μL , respectively. Transport experiments were initiated by adding transport buffer containing the test compounds (1 μM) with 1% (v/v) dimethylsulfoxide (DMSO) to the AP or BL sides. Quinidine and verapamil were used as positive controls (P-gp substrates), and sertraline was used as a negative control (non-Pgp substrate). Just after initiation, 10 μL samples were taken from the AP or BL side in order to determine the initial concentration (C_0). After 20-, 40-, and 60-min incubations at 37°C, 100 μL aliquots were collected from the opposite side and replaced with 100 μL of transport buffer. The concentrations of compounds were determined as described below. AP to BL and BL to AP apparent permeability coefficients (P_{app}) were calculated using Eq. 1-1.

$$P_{app} = (dQ/dt)/(A \times C_0) \quad (\text{Eq. 1-1})$$

where dQ/dt is the permeability rate and A is the surface area of the filter membrane (0.7 cm^2).

The flux ratio was calculated using Eq. 1-2.

$$\text{Flux ratio} = P_{app, \text{BL to AP}} / P_{app, \text{AP to BL}} \quad (\text{Eq. 1-2})$$

where $P_{app, \text{AP to BL}}$ and $P_{app, \text{BL to AP}}$ are AP to BL and BL to AP apparent permeability coefficients, respectively. The net flux ratio was calculated by Eq. 1-3.

$$\text{Net flux ratio} = \text{flux ratio in LLC-GA5-COL150 cells} / \text{flux ratio in LLC-PK1 cells} \quad (\text{Eq. 1-3})$$

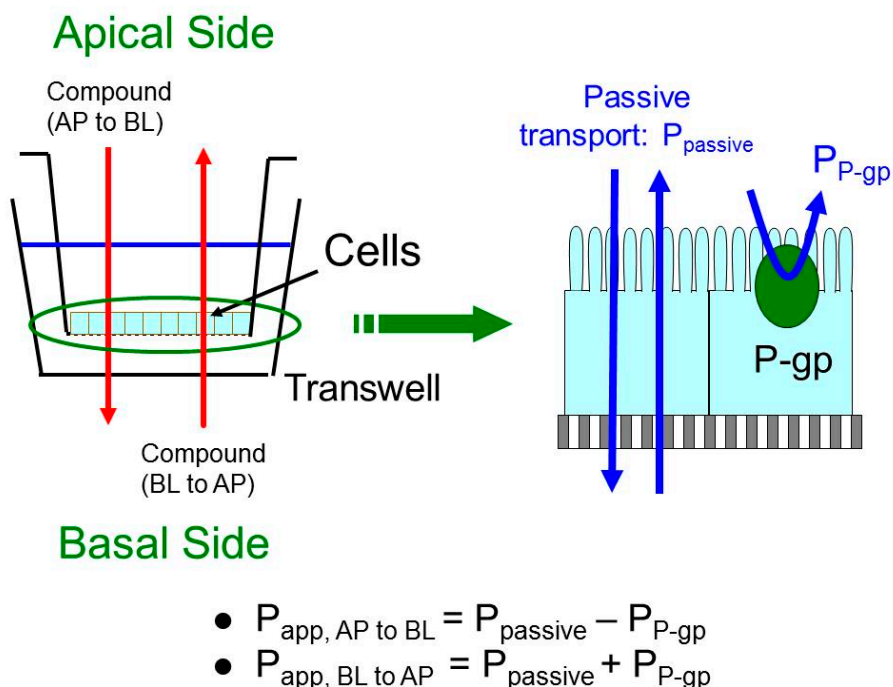


Figure 1-2. Overview of the bidirectional transport assay across LLC-PK1 and LLC-GA5-COL150 cells.

1-2-5. Influence of tebufenozide and methoxyfenozide on quinidine transport by

P-glycoprotein

Cells were seeded as described above. The AP to BL transport of quinidine was evaluated in the absence and presence of various concentrations of tebufenozide or methoxyfenozide. The concentration of quinidine was set at 1 μ M, which was less than the K_m value (30.8 μ M, data not shown). Elacridar (2 μ M), a potent P-gp inhibitor, was used as the positive control. The final concentration of DMSO was adjusted at 1 % (v/v) in the solutions in the AP and BL sides. Cells were washed 2 times with transport buffer, and then preincubated in transport buffer at 37°C. After a 15-min pre-incubation, media in both sides were replaced with transport buffer with or without a test compound (tebufenozide, methoxyfenozide, or elacridar). After a pre-incubation for 30 min, transport experiments were initiated by adding 1 μ M quinidine to the AP side. Just after initiation, 10 μ L samples were taken from the AP side in order to determine the initial concentration of quinidine (C_0). After 60 min, 100 μ L aliquots were collected from the BL side. The concentration of quinidine was determined, and $P_{app, AP \text{ to BL}}$ was calculated by Eq. 1-1. Inhibitory activity [Inhibition %] was calculated by Eq. 1-4.

$$[\text{Inhibition \%}] = (P_{app, AP \text{ to BL, quinidine}} - P_{app, AP \text{ to BL, control}}) / (P_{app, AP \text{ to BL, elacridar}} - P_{app, AP \text{ to BL, control}}) \times 100 \quad (\text{Eq. 1-4})$$

where $P_{app, AP \text{ to BL, quinidine}}$, $P_{app, AP \text{ to BL, control}}$, and $P_{app, AP \text{ to BL, elacridar}}$ are AP to BL apparent permeability coefficient of quinidine with and without a test compound and with elacridar (positive control, 100 % inhibition), respectively. The apparent IC_{50} values of DBHs, the concentrations producing 50% inhibition of the maximum quinidine

transport, were calculated using Phoenix Winnonlin 6.3 (Certara, L.P., NJ, USA).

1-2-6. Pharmacokinetic study in rats after single intravenous administration of methoxyfenozide, elacridar or valspodar

Methoxyfenozide, elacridar, or valspodar dissolved in DMSO were injected into the tail vein of non-fasted rats at 1 mg/0.2 mL/kg. Blood samples were collected from the jugular vein at 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8 and 10 hr for methoxyfenozide and elacridar, and at 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 hr for valspodar. Blood samples were centrifuged at 4°C and 1,880 x g for 15 min in order to obtain plasma. Plasma samples were frozen at -30°C until used. The concentrations of these compounds were determined by the analysis method described in 1-2-8. The plasma concentration-time profiles of methoxyfenozide, elacridar, or valspodar were analyzed using two-compartment model in order to obtain their pharmacokinetic parameters. The analyses were performed with Phoenix Winnonlin 6.3 (Certara, L.P., NJ, USA). The calculated pharmacokinetic parameters were described below.

C_0 : initial concentration, $t_{1/2}$: terminal half-life, AUC_t : area under the concentration-time curve from zero time to the last observed time, AUC_{∞} : AUC extrapolated to infinity, CL: total body clearance, V_{ss} : volume of distribution at steady state, V_1 : volume of distribution for the central compartment, V_2 : volume of distribution for the peripheral compartment, k_{el} : elimination rate constant, k_{12} : transfer rate constant from the central to peripheral, k_{21} : transfer rate constant from the peripheral back to the central

Based on the obtained pharmacokinetic parameters, and Eqs. 1-5 and 1-6, the bolus intravenous doses (D) and infusion rates (k_0) of methoxyfenozide, elacridar, or valspodar were determined for the *in vivo* study to evaluate the brain to plasma concentration ratio of methoxyfenozide in the presence or absence of P-gp inhibitors.

$$C_{ss} = k_0 / (V \times k_{el}) \quad (\text{Eq. 1-5})$$

$$D = k_0 / k_{el} \quad (\text{Eq. 1-6})$$

1-2-7. Determination of the brain to plasma concentration ratio of methoxyfenozide in the presence or absence of P-glycoprotein inhibitors in rats

Under isoflurane anesthesia, the right jugular vein of non-fasted male rats was cannulated with a polyethylene tube (outer diameter 0.80 mm, Natsume Seisakusho, Tokyo, Japan). Methoxyfenozide was injected into the rats followed by a continuous infusion for 3 hr in order to obtain steady state plasma concentrations in the absence or presence of the P-gp inhibitors (elacridar and valspodar). Bolus intravenous doses and infusion rates were as follows: 0.15 mg/kg and 0.5 mg/hr/kg for methoxyfenozide, 4 mg/kg and 2 mg/hr/kg for elacridar, and 2.5 mg/kg and 1 mg/hr/kg for valspodar, respectively. DMSO/polyethylene glycol/phosphate-buffered saline (PBS) (10:60:30, v/v) was used as the dosing vehicle for bolus injections, and DMSO/polyethylene glycol/PBS (5:60:35, v/v) for continuous infusions. Blood samples were collected from the left jugular vein at 1, 2, and 3 hr, and centrifuged at 4°C and 1,880 x g for 15 min in order to obtain plasma. Immediately after the last blood sampling, rats were sacrificed by exsanguination, and the brain was collected. The brain was homogenized in a 3-fold volume of PBS to obtain a 25% brain homogenate. Plasma samples and 25% brain homogenate were frozen at -30°C until used. The concentrations of the compounds were determined as described below, and $K_{p, \text{brain}}$ values were obtained by dividing the brain concentration by the plasma concentration at the last sampling point.

1-2-8. Quantification

The concentrations of tebufenozide, methoxyfenozide, elacridar, valspodar, quinidine, verapamil, and sertraline were determined using liquid chromatography with tandem mass spectrometry. The samples obtained from the transport study were mixed with 1.5 volumes of acetonitrile and 0.1 volumes of 5 µg/mL chlorpropamide as the internal standard (IS), and the mixture was then injected into LC-MS/MS (API 4000 triple quadrupole mass spectrometer with TurboionSpray (AB SCIEX, MA, USA) equipped with the SHIMAZU HPLC system (SHIMAZU, Kyoto, Japan)). Plasma samples were mixed with 15 volumes of acetonitrile/methanol (50/50, v/v) and 0.5 volumes of 5 µg/mL chlorpropamide (IS), respectively. Brain homogenates were mixed with 3 volumes of acetonitrile/methanol (50/50, v/v) and 0.1 volumes of 5 µg/mL chlorpropamide (IS), respectively. They were then centrifuged at 4°C and 20,400 x g for 10 min, and the supernatants obtained were injected into LC-MS/MS.

The HPLC analysis conditions used were as follows: column: Ascentis Express F5 column (Supelco, 2.7 µm particles, 2.1 mm i.d. × 75 mm) for valspodar and Ascentis Express C18 column (Supelco, 2.7 µm particles, 2.1 mm i.d. × 75 mm) for the other compounds, temperature: 40°C, solvent: A: 0.1% (v/v) formic acid; B: 0.1% (v/v) formic acid in acetonitrile, gradient: B: 15% (0 min) - 15% (0.3 min) - 95% (0.3 min) - 95% (2.0 min) for valspodar; B: 5% (0 min) - 5% (0.25 min) - 95% (2.0 min) - 95% (2.5 min) for the other compounds, flow rate: 0.7 mL/min.

The MS/MS analysis conditions used were as follows: detection: multiple reaction monitoring (MRM), ion source: electrospray ionization (ESI), polarity: the negative ion mode for tebufenozide and methoxyfenozide; the positive ion mode for the other compounds, monitor ions (precursor-to-product ion): tebufenozide: 352/149;

methoxyfenozide: 367/149; elacridar: 564/252; valsopodar: 1215/425; quinidine: 325/307; verapamil: 455/165; sertraline: 306/275; chlorpropamide (IS): 277/175 for the positive ion mode and 275/190 for the negative ion mode.

1-2-9. Statistical analysis

All statistical analyses were performed using Microsoft Excel Statistics 2012 for Windows (SSRI Co. Ltd., Tokyo, Japan). The significance of differences in the *in vitro* bidirectional transport assay between LLC-PK1 and LLC-GA5-COL150 cells was analyzed by the Student's two-tailed t test. The significance of differences between groups in the *in vivo* study of rats was analyzed by a one-way analysis of variance followed by Dunnett's test. A value of $P < 0.05$ was considered significant.

1-3. Results

1-3-1. Bidirectional transport assay across LLC-PK1 and LLC-GA5-COL150 cells

The P-gp-mediated transport of tebufenozide and methoxyfenozide using LLC-PK1 and LLC-GA5-COL150 cells was evaluated. Figure 1-3 shows the time courses of their transcellular transport. In LLC-PK1 and LLC-GA5-COL150 cells, the AP to BL and BL to AP transport of tebufenozide and methoxyfenozide was almost linear up to 60 min after the initiation of transport, indicating that initial rates were maintained for at least 60 min. The initial rates of the other compounds (verapamil, quinidine, and sertraline) were also maintained for at least 60 min (data not shown). The apparent permeability and flux ratios of the test compounds were summarized in Table 1-2.

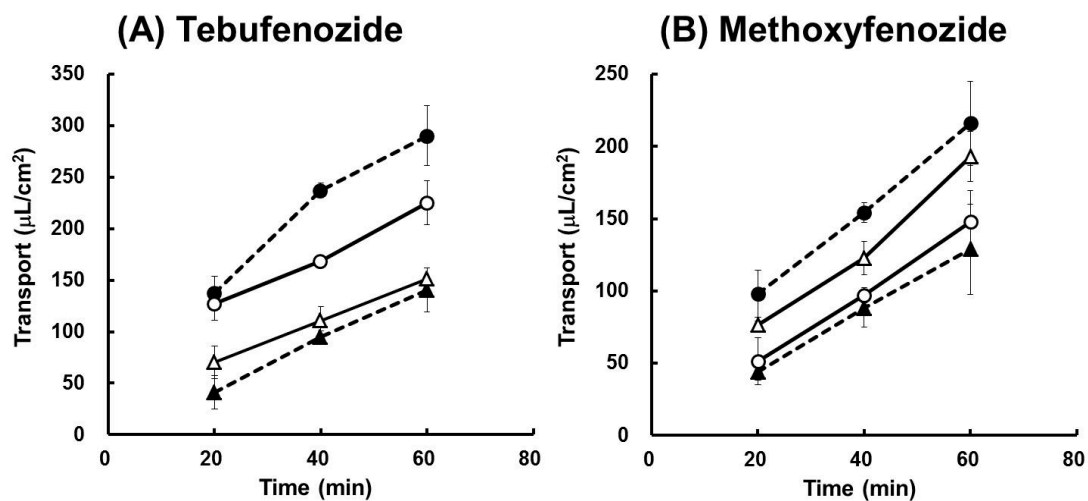


Figure 1-3. Time profiles of transport of (A) tebufenozide (1 μM) and (B) methoxyfenozide (1 μM) across LLC-PK1 cells and LLC-GA5-COL150 in the bidirectional transport assay. ●: AP to BL transport across LLC-PK1, ▲: BL to AP transport across LLC-PK1, ○: AP to BL transport across LLC-GA5-COL150, △: BL to AP transport across LLC-GA5-COL150. Data shown are the mean $\pm$ S.D. (n = 3).

Table 1-2. Bidirectional transport assay using LLC-PK1 and LLC-GA5-COL150 cells.

	LLC-PK1 cells			LLC-GA5-COL150 cells		
	P _{app} , AP to BL (cm/sec, ×10 ⁻⁶)	P _{app} , BL to AP (cm/sec, ×10 ⁻⁶)	Flux Ratio	P _{app} , AP to BL (cm/sec, ×10 ⁻⁶)	P _{app} , BL to AP (cm/sec, ×10 ⁻⁶)	Flux Ratio
Tebuufenozide	63.7 ± 17.1	41.4 ± 2.1	0.65	40.7 ± 8.7	33.7 ± 10.6	0.83
Methoxyfenozide	49 ± 5.3	38.3 ± 14.9	0.78	40.2 ± 7.1	48.4 ± 7.4	1.2
Quinidine	38.7 ± 4.3	37.7 ± 4.3	0.97	2.14 ± 0.33**	88.3 ± 3.5**	41
Verapamil	43.4 ± 6	26.5 ± 8.4	0.61	17.2 ± 0.9*	63.2 ± 11.1*	3.7
Sertraline	4.62 ± 1.82	4.14 ± 0.49	0.9	5.42 ± 1.77	3.49 ± 0.51	0.64
						0.72

Concentrations of test compounds are set at 1 μM. The significance of differences in Papp values between LLC-PK1 cells and LLC-GA5-COL150 cells was analyzed (*: $p < 0.05$, **: $p < 0.01$). Data shown are the mean ± S.D. (n =3).

In the bidirectional transport assay, compounds are classified into P-gp transport substrates if their net flux ratio in MDR-1 overexpressing epithelial cells or flux ratio in Caco-2 cells is equal to or more than 2, whereas compounds are regarded as weak or non-P-gp transport substrates if their net flux ratio or flux ratio is smaller than 2 (FDA Guidance, 2012; EMA Guideline, 2012; Polli *et al.*, 2001; Fekete *et al.*, 2015). Regarding the positive control (quinidine and verapamil), BL to AP transport was higher than AP to BL transport in LLC-GA5-COL150 cells transfected with human *MDR1* (net flux ratios of quinidine and verapamil: 42 and 6.0, respectively), while transport in both directions was almost the same in LLC-PK1 cells. On the other hand, no significant difference was observed in BL to AP or AP to BL transport in either cell with the negative control (sertraline) (net flux ratio: 0.72). The flux ratios of tebufenozide and methoxyfenozide were 0.65 and 0.78, respectively, in LLC-PK1 cells, and 0.83 and 1.2, respectively, in LLC-GA5-COL150 cells. The net flux ratios of tebufenozide and methoxyfenozide were 1.3 and 1.5, respectively. Therefore, tebufenozide and methoxyfenozide were considered to be weak or non-P-gp substrates based on these results.

1-3-2. Influence of tebufenozide and methoxyfenozide on quinidine transport by

P-glycoprotein

In order to determine the inhibitory effects of DBHs on P-gp, we examined the influence of tebufenozide and methoxyfenozide on the AP to BL transport of quinidine using LLC-GA5-COL150 cells. The flux ratio of BL to AP transport/AP to BL transport of quinidine in LLC-PK1 cells was less than 1.0, indicating that quinidine did not undergo transporter-mediated efflux in LLC-PK1 cells. Therefore, the net flux ratio was not used, but $P_{app, AP \text{ to } BL}$ in LLC-GA5-COL150 cells was employed in order to evaluate

the inhibitory effects of tebufenozide and methoxyfenozide on quinidine transport by P-gp. Concentration-dependent inhibitory effects were observed (Figure 1-4), and IC_{50} values were calculated. The IC_{50} values of tebufenozide and methoxyfenozide were 21.5 and 44.3 μM , respectively.

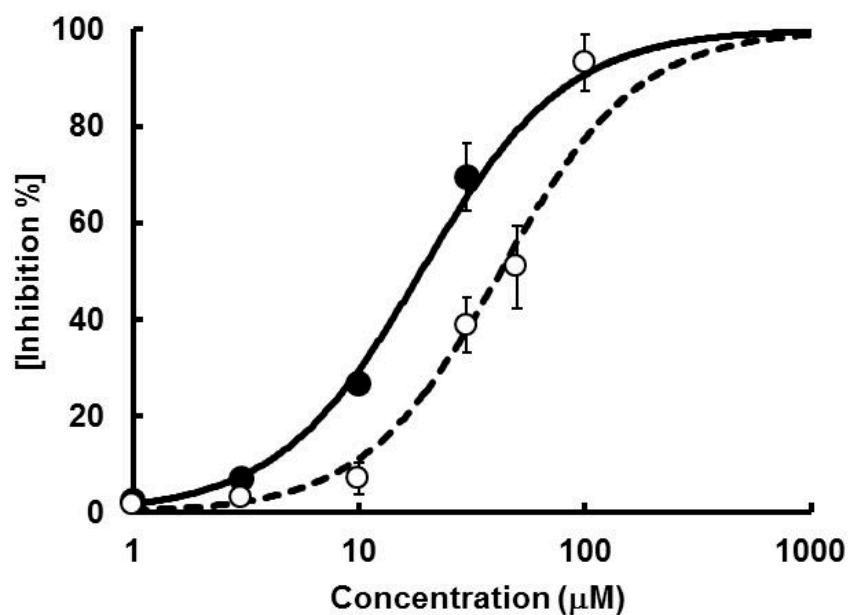


Figure 1-4. Influence of tebufenozide and methoxyfenozide on quinidine transport by P-gp. The $P_{app, AP \text{ to } BL}$ of quinidine (1 μM) was evaluated in the presence of various concentrations of tebufenozide (●) or methoxyfenozide (○). Data shown are the mean $\pm$ S.D. (n=3).

1-3-3. Pharmacokinetic study in rats after single intravenous administration of methoxyfenozide, elacridar or valspodar

The plasma concentrations of methoxyfenozide, elacridar, or valspodar were evaluated following a single intravenous administration of methoxyfenozide, elacridar, or valspodar at 1 mg/kg to the male rats, and determined the pharmacokinetic parameters of these compounds. The obtained plasma concentration-time profiles and calculated pharmacokinetic parameters of these compounds were shown in Figure 1-5 and Table 1-3.

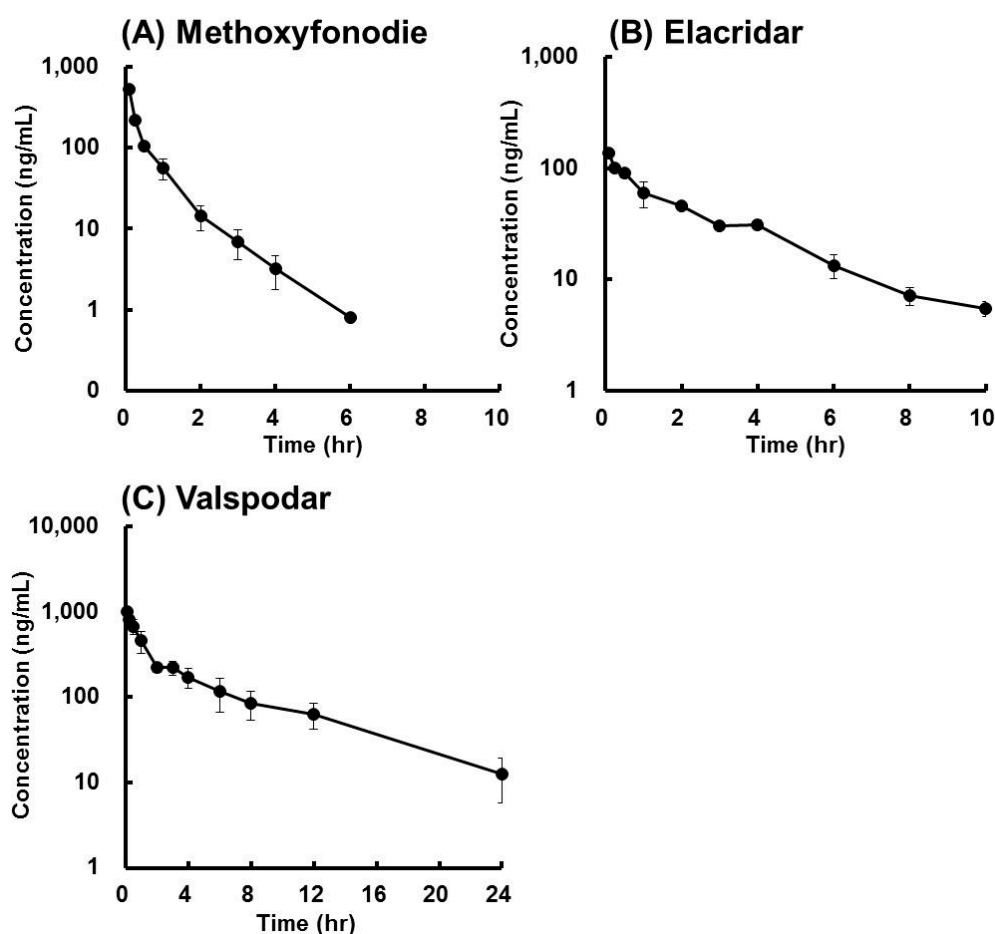


Figure 1-5. Plasma concentration-time profiles of methoxyfenozide (A), elacridar (B), and valspodar (C). Methoxyfenozide, elacridar, and valspodar were intravenously administrated to non-fasted male rats at 1 mg/ kg, respectively. Data shown are the mean ± S.D. (n =3).

Table 1-3. Pharmacokinetic parameters of methoxyfenozide, elacridar, and valsopodar.

Compound	Animal No.	C ₀ ng/mL	AUC _t ng·hr/mL	AUC _∞ ng·hr/mL	t _{1/2} hr	CL mL/hr/kg	V _{ss} mL/kg	V ₁ mL/kg	V ₂ mL/kg	k _{e1} 1/hr	k ₁₂ 1/hr	k ₂₁ 1/hr
Methoxyfenozide	1	809	227	229	0.838	4369	2531	937	1456	4.58	5.22	3.36
	2	763	246	251	1.51	3982	3590	1278	1935	3.18	2.51	1.66
	3	856	238	242	0.844	4127	2595	1109	1438	3.79	1.94	1.49
	Average	809	237	241	1.065	4159	2905	1108	1610	3.85	3.23	2.17
	SD	47	10	11	0.388	195	594	170	282	0.70	1.75	1.03
Elacridar	1	147	302	323	2.60	3092	10894	6420	4126	0.485	2.34	3.63
	2	179	278	293	2.36	3416	11166	5805	5972	0.579	1.60	1.55
	3	149	280	296	2.49	3376	10474	6961	3541	0.482	0.64	1.26
	Average	158	287	304	2.48	3295	10844	6395	4546	0.516	1.52	2.15
	SD	18	13	17	0.12	177	349	578	1269	0.055	0.85	1.30
Valsopodar	1	1411	2642	2701	4.36	370	2219	842	1561	0.432	0.950	0.512
	2	884	3012	3189	6.13	314	2341	1048	1336	0.297	0.459	0.360
	3	1084	2125	2196	5.85	455	2405	924	1179	0.497	0.493	0.387
	Average	1126	2593	2695	5.44	379.7	2322	938	1359	0.408	0.634	0.420
	SD	266	446	497	0.95	71.4	94	103	192	0.102	0.274	0.081

Methoxyfenozide, elacridar, and valsopodar were intravenously administered to non-fasted male rats at 1 mg/ kg, respectively.

Plasma concentration-time profile of methoxyfenozide, elacridar, or valsopodar was analyzed using 2-compartment model.

1-3-4. Determination of the brain to plasma concentration ratio of methoxyfenozide in the presence or absence of P-glycoprotein inhibitors in rats

The influence of P-gp inhibitors on the brain disposition of methoxyfenozide in rats was evaluated in order to examine the P-gp-mediated transport of DBHs *in vivo*. Since methoxyfenozide has higher aqueous solubility than that of other DBH derivatives, it was selected for the *in vivo* study. Methoxyfenozide was intravenously administered to rats followed by its continuous infusion for 3 hr in the absence and presence of elacridar or valspodar (P-gp inhibitors). The plasma concentrations of methoxyfenozide, elacridar and valspodar are shown in Figure 1-6.

The plasma concentrations of methoxyfenozide, elacridar and valspodar reached a plateau 2 hr after their administration, indicating that the steady state plasma concentrations of these compounds were achieved at least 2 hr after their administration. Thus, the brain concentration of methoxyfenozide was determined 3 hr after its administration, and the $K_{p,brain}$ values of methoxyfenozide were obtained at the steady state. The $K_{p,brain}$ values obtained in the absence or presence of P-gp inhibitors are shown in Figure 1-7. The $K_{p,brain}$ value in the absence of P-gp inhibitors was 1.5, while those in the presence of elacridar and valspodar were 2.8 and 2.3, respectively. The $K_{p,brain}$ value of methoxyfenozide significantly increased in the presence of elacridar or valspodar, suggesting that methoxyfenozide is a P-gp transport substrate.

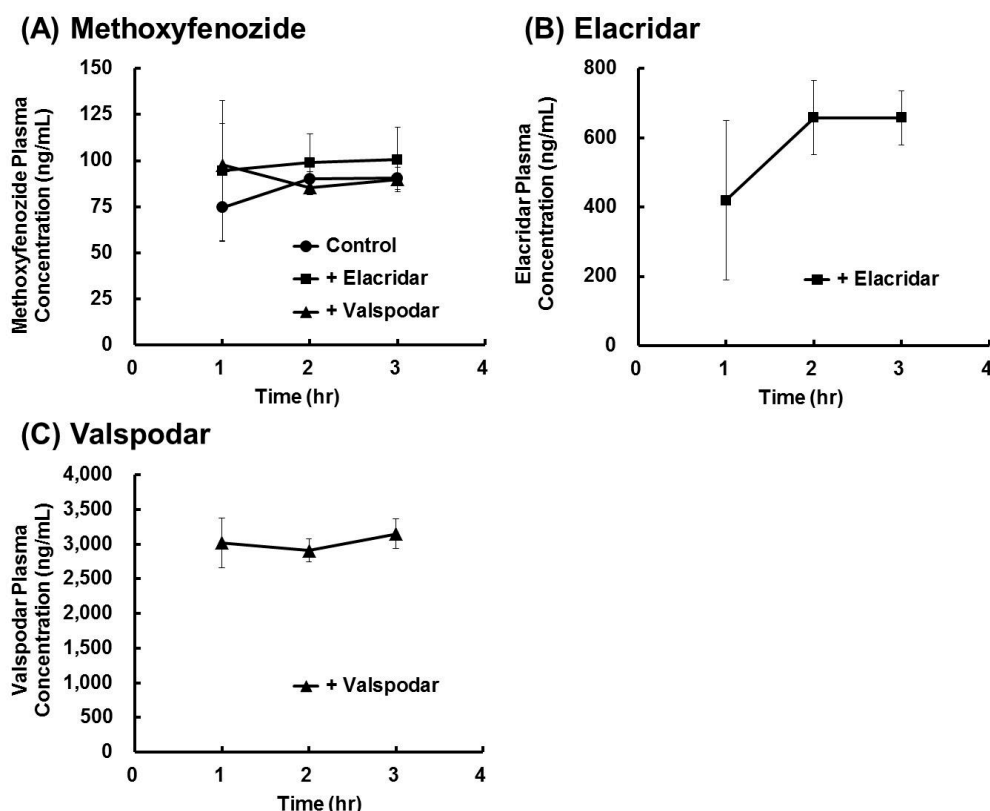


Figure 1-6. Plasma concentration-time profiles of (A) methoxyfenozide, (B) elacridar, and (C) valspodar after bolus injections followed by the intravenous infusion of methoxyfenozide in the absence (●, control) and presence of elacridar (■) or valspodar (▲). Bolus intravenous doses and infusion rates were as follows: 0.15 mg/kg and 0.5 mg/hr/kg for methoxyfenozide, 4 mg/kg and 2 mg/hr/kg for elacridar, and 2.5 mg/kg and 1 mg/hr/kg for valspodar, respectively. Data shown are the mean $\pm$ S.D. ($n=3$).

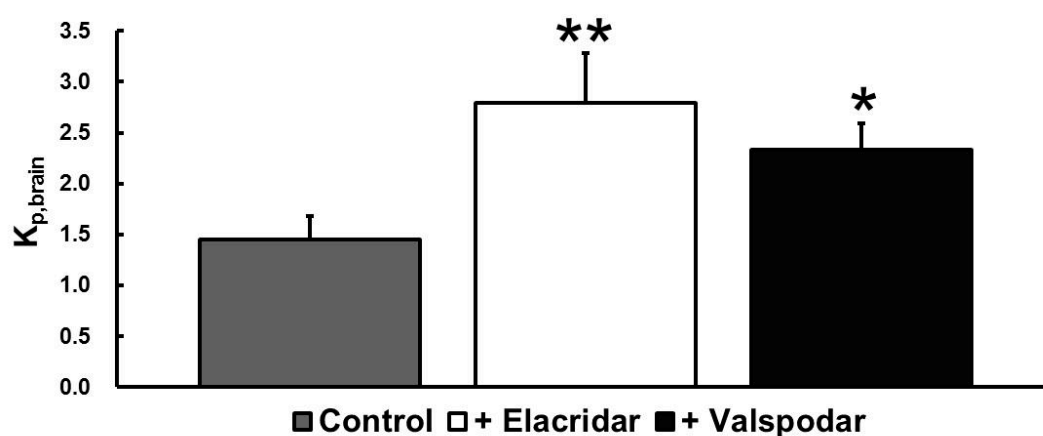


Figure 1-7. $K_{p,brain}$ values of methoxyfenozide after bolus injections followed by the intravenous infusion of methoxyfenozide in the absence (control) and presence of elacridar or valspodar. $K_{p,brain}$ values were significantly increased in the presence of elacridar or valspodar. (*: $p < 0.05$, **: $p < 0.01$. Data shown are the mean $\pm$ S.D. ($n=3$)).

1-4. Discussion

The cell-based bidirectional transport assay has been recommended as the method to identify P-gp transport substrates and inhibitors by the Food and Drug Administration (FDA) (FDA guidance on drug interaction studies in 2012 (FDA Guidance, 2012)) and European Medicines Agency (EMA) (EMA guidelines on drug interaction studies in 2012 (EMA Guideline, 2012)). Moreover, several studies have shown a stronger correlation of *in vivo* P-gp function at BBB (the ratios of $K_{p,brain}$ values in P-gp knockout mice to $K_{p,brain}$ values in wild-type mice) with the *in vitro* net flux ratio in the bidirectional transport assay than with the ATPase activity assay or the uptake inhibition assay (Feng *et al.*, 2008; Adachi *et al.*, 2001). Uchida *et al.* (2011, 2014) estimated $K_{p,brain}$ values from the findings of the bidirectional transport assay in mice and monkeys, and obtained good predictions. Thus, the bidirectional transport assay is regarded as the definitive *in vitro* method to identify P-gp substrates. However, the bidirectional transport assay may also fail to identify substrates with high passive permeability because of the masking the P-gp function by their high passive permeabilities (Polli *et al.*, 2001; von Richter *et al.*, 2009; Fekete *et al.*, 2015).

Animal studies are useful for elucidating the *in vivo* physiological function of P-gp, and may be used to evaluate the impact of P-gp on the human pharmacokinetics of compounds; however, concerns have been expressed regarding species differences between human MDR1 and rat or mouse *mdr1a/1b*. To date, several studies have examined species differences in P-gp (Feng *et al.*, 2008; Katoh *et al.*, 2006; Takeuchi *et al.*, 2006; Yamazaki *et al.*, 2001; Bundgaard *et al.*, 2012). Feng *et al.* (2008) demonstrated that species differences between human and mouse P-gp were relatively rare based on a correlation analysis of the net flux ratio of 3,300 compounds using the

bidirectional transport assay with *MDR1*- and *mdr1a*-transfected cells (coefficient of determination $r^2 = 0.92$). Takeuchi *et al.* (2006) conducted similar correlation analyses to Feng *et al.* for the flux ratio between human *MDR1*- and mouse or rat *mdr1a*-transfected cells for 12 compounds ($r^2 = 0.76$ for rats, $r^2 = 0.61$ for mice). Regarding species difference between rats and mice, previous studies reported similar functions for P-gp (Takeuchi *et al.*, 2006; Bundgaard *et al.*, 2012). Thus, no prominent species differences exist for P-gp function. Furthermore, the linear correlation slope was found to not equal one, but was higher in a correlation analysis between the ratios of $K_{p,brain}$ values in P-gp knockout mice to those in wild-type mice, and the net flux ratio in the bidirectional transport assay (Feng *et al.*, 2008; Adachi *et al.*, 2001). One of the reasons for this higher slope may be higher P-gp expression levels in BBB than in cells. These findings indicate that an *in vivo* animal study is a more sensitive method for identifying P-gp transport substrates than *in vitro* methods.

A concern associated with inhibition studies using P-gp inhibitors *in vivo* in rats is that other efflux transporters such as Mrp4 and Bcrp are also expressed in the rat BBB (Hoshi *et al.*, 2013), and DBHs may be transported by Mrp and/or Bcrp as well as P-gp. In rats, elacridar is known not only as a potent P-gp inhibitor (K_i : 0.0016 μM) (Sugimoto *et al.*, 2011), but also as a potent Bcrp inhibitor (IC_{50} : 0.0941 μM) (Hartz *et al.*, 2010), while valspodar is recognized as a potent P-gp inhibitor (IC_{50} : 0.03 μM) (Milane *et al.*, 2011). However, a previous study reported that valspodar inhibited rat Bcrp (IC_{50} : >10 μM) (Muenster *et al.*, 2008). The $[I_u]$ (unbound plasma concentration of inhibitors) / K_i (or IC_{50}) value has frequently been used as the indicator showing the greatest likelihood of *in vivo* inhibition (FDA Guidance, 2012; EMA guideline, 2012). If a compound has a higher $[I_u / K_i \text{ (or } \text{IC}_{50})]$ value for an efflux transporter than another

efflux transporter, it indicates that the compound more strongly inhibits the function of the former efflux transporter. The estimated $[I_u / K_i \text{ (or } IC_{50})]$ values of elacridar and valspodar for P-gp and Bcrp in an *in vivo* study in rats were listed in Table 1-4. The larger $[I_u / K_i \text{ (or } IC_{50})]$ values of both compounds for P-gp show that they inhibit P-gp more strongly than Bcrp under these conditions. Moreover, Hoshi *et al.* (2013) showed that the expression level of P-gp was 4.6-fold and 12-fold higher than those of Bcrp and Mrp4, respectively, in the rat BBB using LC-MS/MS methods, and, as such, P-gp is the most strongly expressed efflux transporter in the rat BBB. Therefore, P-gp rather than Bcrp and Mrp is likely to limit the distribution of DBHs in the rat brain when their distribution in the brain is inhibited by elacridar and valspodar.

In the present study, the net flux ratio of tebufenozide and methoxyfenozide was measured using the cell-based bidirectional transport assay; however, the results obtained suggested that these compounds were likely to be weak or non-P-gp substrates. Fujikawa *et al.* (2007) found that the $P_{app, AP \text{ to } BL}$ of tebufenozide and methoxyfenozide in Caco-2 cells were 36×10^{-6} and 47×10^{-6} cm/sec, which were higher than that of metoprolol, a high passive permeability marker compound, 23×10^{-6} cm/sec. Therefore, the absence of the P-gp-mediated efflux of DBH derivatives may be due to the high passive transcellular permeability of these compounds.

Table 1-4. Estimation of $[I_u / (K_i \text{ or } IC_{50})]$ values of elacridar and valspodar in rats.

Inhibitor	<i>In vitro</i> rat K_i or IC_{50} (μ M)		Unbound fractions in rat plasma	I (μ M) ^a	I_u (μ M) ^b	$I_u / K_i \text{ (or } IC_{50})$	
	P-gp	Bcrp				P-gp	Bcrp
Elacridar	0.0016 ^c	0.0941 ^d	0.019 ^e	1.17	0.0222	14	0.24
Valspodar	0.03 ^f	>10 ^g	0.15 ^h	2.59	0.389	13	<0.039

a: Plasma concentrations of elacridar or valspodar at the steady state, b: $I \times$ unbound fractions in rat plasma, c: Sugimoto *et al.*, 2011, d: Hartz *et al.*, 2010, e: Kallem *et al.*, 2012, f: Milane *et al.*, 2011, g: Muenster *et al.*, 2008, h: Binkhathlan *et al.*, 2010.

The author then evaluated the influence of P-gp inhibitors (elacridar and valspodar) on the brain distribution of methoxyfenozide using rats in order to investigate the P-gp-mediated transport of DBHs *in vivo*. The results obtained suggested that methoxyfenozide is a P-gp transport substrate. Fekete *et al.* previously showed that compounds having high passive permeability such as ketoconazole and itraconazole were not transported by P-gp in the *in vitro* bidirectional transport assay; however, these compounds stimulated P-gp ATPase activity and were transported by P-gp *in vivo* (Fekete *et al.*, 2015). Thus, an *in vivo* study may be more sensitive than *in vitro* assays in determining whether compounds having high passive permeability are P-gp transport substrates.

Based on a comprehensive evaluation of the results together with previous ATPase activity findings (Kanaoka *et al.*, 2013), it was concluded that DBHs such as tebufenozide and methoxyfenozide are weak P-gp transport substrates, and they are excreted by P-gp using the ATP hydrolysis energy although the efflux is masked by their high passive transport in the *in vitro* bidirectional transport assay. Thus, there is a possibility that other P-gp inhibitors may increase the concentration of DBHs in the human tissues where P-gp expresses. However, the risk that increased DBHs concentrations cause side effects are considered to be low, because DBHs are weak P-gp substrates and safe pesticides even if a small amount of them would be absorbed as pesticide residues.

The author also investigated the inhibitory effects of tebufenozide and methoxyfenozide on quinidine transport by P-gp in an attempt to ascertain whether these derivatives are P-gp inhibitors. Concentration-dependent inhibitory effects (IC₅₀ values: tebufenozide 21.5 μ M, methoxyfenozide 44.3 μ M) were observed. The IC₅₀

values of these DBHs were larger than those of verapamil (IC_{50} value: 3.92 μ M) and cyclosporine A (IC_{50} value: 1.51 μ M) (Patil *et al.*, 2011), which are potent P-gp inhibitors, and were similar to that of clarithromycin (IC_{50} value: 34-66 μ M) (Cook *et al.*, 2010; Mueck *et al.*, 2013), which is a moderate P-gp inhibitor. Therefore, tebufenozide and methoxyfenozide were moderate P-gp inhibitors. According to the FDA guidance (2012), if the $[I_u / K_i \text{ (or } IC_{50})]$ value of a compound for P-gp is estimated to be smaller than 0.1, an *in vivo* drug interaction study on the compound is not required. The predicted unbound fractions of tebufenozide and methoxyfenozide from their structures in human plasma were 0.0298 and 0.0382, respectively (ADMET Predictor v7.2, Simulations Plus, Inc., CA, USA). Using these values and IC_{50} values, the human plasma concentrations of tebufenozide and methoxyfenozide required for being the $[I_u / K_i \text{ (or } IC_{50})] = 0.1$ are estimated to be 72.1 and 116 μ M, respectively. However, it is unlikely that humans are exposed to such high concentrations of the DBHs even if they are taken into the body as pesticide residues in food. Thus, the risk of the DBHs influencing the absorption and disposition of other chemicals including drugs is considered to be low as long as DBHs are properly used.

CHAPTER 2:

Structure-activity relationships of dibenzoylhydrazines for the inhibition of human P-glycoprotein-mediated quinidine transport

2-1. Introduction

The 3D structure of human P-gp has not been clarified yet because there is no crystal structure for human P-gp at present. However, an X-ray crystal structure for mouse P-gp in the inward-facing conformation, which has 87% sequence identity to human P-gp, has been reported previously (Aller *et al.*, 2009; Szewczyk *et al.*, 2015; Li *et al.*, 2014). The inward-facing conformation, in which the substrate-binding site is open to the cytoplasm, enables substrates to access binding sites from the inner leaflets (cytosolic side) of the membranes (Figure 2-1) (Aller *et al.*, 2009; Szewczyk *et al.*, 2015; Li *et al.*, 2014).



Figure 2-1. X-ray crystal structure of the complex of mouse P-gp in the inward-facing conformation with QZ-Ala (a cyclic peptide) (PDB: 4Q9I).

Various models have been proposed in order to explain the transport mechanism used by P-gp. These models suggest that substrates are initially partitioned into a lipid bilayer, and then bind to the transmembrane domain of the inward-facing conformation from the cytosolic side of the membrane (Aller *et al.*, 2009; Clay and Sharom, 2013; Li *et al.*, 2014; Sharom, 2014; Gutmann *et al.*, 2010). This binding triggers a structural change to the outward-facing conformation of P-gp, which exposes the binding site to the extracellular side. Substrates are then excluded as a result of decreased binding affinity. This structural conversion requires the hydrolysis of ATP, which binds to the interface of the two NBDs of P-gp (Aller *et al.*, 2009; Li *et al.*, 2014).

P-gp substrates generally appear to be hydrophobic, and have a large molecular volume, electronegative groups, and hydrogen bonding groups (Wang *et al.*, 2003; Seelig, 1998). Previous studies have been conducted in order to investigate the mechanisms underlying P-gp substrate/inhibitor recognition using QSAR (Chen L *et al.*, 2012). However, these recognition mechanisms have not yet been elucidated in detail.

In this chapter, DBHs with structurally diverse substituents on the B ring was used

as model compounds in order to investigate the mechanisms responsible for P-gp substrate/inhibitor recognition. The author evaluated their inhibitory activities on quinidine transport by human P-gp using MDR1-LLC-PK1 cells as an index of the strength of the DBH-P-gp interaction. The inhibitory activities of the two series of DBHs, 18 compounds having 3,5- dimethyl groups (Series I) and 40 compounds having 2-Cl groups (Series II) on the A-ring, were measured. The structure-inhibitory activity relationship was then investigated using a classical QSAR analysis and one of 3D-QSAR, comparative molecular field analysis (CoMFA) (Cramer *et al.*, 1988) to evaluate substituent effects on P-gp recognition.

2-2. Materials and Methods

2-2-1. Chemicals

Tebufenozide and methoxyfenozide were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Quinidine was obtained from Sigma-Aldrich (MO, USA). Other DBHs were synthesized and reported in previous studies by Nakagawa's group (Oikawa *et al.*, 1994; Wheelock *et al.*, 2006). FBS was purchased from the American Type Culture Collection (ATCC, MD, USA). All other chemicals were of analytical grade, and purchased either from Sigma-Aldrich (MO, USA) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2-2-2. Cell culture

LLC-GA5-COL150 cells (LLC-PK1 porcine kidney epithelial cells transfected with human *MDR1*) (Tanigawara *et al.*, 1992; Ueda *et al.*, 1992) were obtained from the RIKEN BioResource Center (Ibaraki, Japan). LLC-GA5-COL150 cells were cultured according to the methods described in 1-2-3.

2-2-3. P-glycoprotein inhibition study using LLC-GA5-COL150 cells

The inhibitory activities of DBHs on quinidine transport by human P-gp were evaluated according to the methods described in 1-2-5. The AP to BL transport of quinidine was evaluated in the absence and presence of a test compound. The concentrations of quinidine and DBHs were set at 1 μ M and 30 μ M, respectively. Elacridar (2 μ M), a potent P-gp inhibitor, was used as the positive control. The concentration of quinidine was determined, and the AP to BL apparent permeability coefficient (P_{app}) was calculated using Eq. 1-1. Inhibitory activity [Inhibition %] was calculated by Eq. 1-4. The P_{app} in LLC-GA5-COL150 cells was used to evaluate the inhibitory effects of DBHs on quinidine transport by P-gp as described in 1-3-2.

2-2-4. Quantification of quinidine concentrations

The concentration of quinidine was determined according to the method described in 1-2-8.

2-2-5. Logit transformation of inhibitory activities for the classical QSAR and

CoMFA analyses

Inhibitory activity was logit-transformed for the classical QSAR and CoMFA analysis by Eq. 2-1.

$$\text{logit A} = \log [\text{Inhibition \%} / (100 - \text{Inhibition \%})] \quad (\text{Eq. 2-1})$$

Compounds having too low (less than 3%) and too high (more than 97%) inhibitory activities were not included in the analyses because the logit function with a sigmoid curve had large errors at both ends. Thus, 5 of 18 compounds of Series I and 3 of 40 compounds of Series II were excluded from the analyses (Table 2-1).

2-2-6. Classical QSAR analysis

A classical QSAR analysis was performed using Microsoft Excel Statistics 2012 for Windows (SSRI Co. Ltd., Tokyo, Japan). The Log P (P : partition coefficient in 1-octanol/water system) values of compounds as a hydrophobicity parameter were taken from the literature (Oikawa *et al.*, 1994). The STERIMOL length parameter L , which represents the length of the substituent along the axis of a bond between the parent molecule and substituent, was used as the steric parameter (Hansch *et al.*, 1995; Verloop *et al.*, 1976). The Swain-Lupton field constant F , which represents the field effect ($\approx$ inductive effect) of the substituent, was used as the electronic parameter (Hansch *et al.*, 1995). The more electron-withdrawing a substituent is, the larger its F value.

2-2-7. CoMFA analysis

CoMFA analysis was carried out with the molecular modeling software package SYBYL ver. 7.3 (Sybyl Molecular Modeling Software; Tripos Associates, Inc.; MO, USA). The X-ray crystallographic structure of an unsubstituted derivative (Nakagawa *et al.*, 1995) was modified to construct the tebufenozide structure. It was energy minimized by Tripos force fields and then used as a template. The structures of the other DBHs were constructed by modifying the structure of tebufenozide. The electrostatic potential (esp) charges of MNDO were calculated for the energy-minimized structure of compounds. Compounds were automatically aligned with the common skeletal chain (C-CO-N-N-CO-C) as a template using the SYBYL module, Align Database. CoMFA was performed for logit-transformed inhibitory activities (logit A). Analyses were conducted with the “Advanced CoMFA” module of SYBYL. The procedure was similar to that described in a previous report (Tamura *et al.*, 2006). Superimposed sets of stable

conformers were placed in a lattice of $23.9 \text{ \AA} \times 23.2 \text{ \AA} \times 22.8 \text{ \AA}$ ($X = -13.9$ to 10.0 , $Y = -10.3$ to 12.9 , $Z = -8.7$ to 14.1) with $2 \text{ \AA}$ spaces automatically generated by the CoMFA routine in SYBYL. The potential energy fields of each stable conformer were calculated at the lattice intersections. To calculate the Coulombic electrostatic potential at each lattice point, the charge of $+1.0$ as a probe and the atomic charges for each of the molecules were used. The steric interaction (Lennard-Jones) potential at the lattice points was calculated using the sp^3 -carbon atom as a probe. The data matrix was analyzed by the partial least squares method (Lindberg *et al.*, 1983). The results of the analysis were expressed as correlation equations with the number of latent variable terms, each of which was a linear combination of original independent lattice variables. In order to show favorable and unfavorable potential regions, variables were displayed as contour diagrams of coefficients of the corresponding field descriptor terms at each lattice intersection. The number of compounds in the set was initially selected as the number of the cross-validation (the leave-one-out method) and then performed an analysis using the optimum number of latent variables deduced from the cross-validation tests without actual cross-validation.

2-3. Results

2-3-1. Inhibitory activities of DBHs against P-glycoprotein -mediated quinidine transport

The author used logit A values transformed from the inhibitory activities [Inhibition %] of DBHs at $30 \text{ }\mu\text{M}$ against maximum quinidine transport as the index of inhibitory activity because it was difficult to determine apparent IC_{50} values, the concentration producing 50% inhibition of the maximum quinidine transport, for DBHs

with poor water solubility. The IC₅₀ values measured for relatively hydrophilic DBHs are listed in Table 2-1. Logit A values positively correlated with -log (IC₅₀) values for 14 DBHs ($r^2 = 0.838$) as shown in Figure 2-2. Thus, it was considered reasonable to use logit A instead of IC₅₀.

The inhibitory activities of the two series of DBHs, 18 compounds of Series I and 40 compounds of Series II were determined using LLC-GA5-COL150 cells. All compounds were neutral under the experimental conditions used. Log *P* values ranged between 3.34 (**4**) and 4.91 (**9**) among the Series I, and between 1.99 (**25**) and 4.29 (**58**) among the Series II. Inhibitory activities ranged between 0.5% (**13**) and 58.4% (**8**) for the Series I, and between 1.7% (**25**) and 98.9% (**56**) for the Series II. The mean and median values of inhibitory activities were 27.8% and 31.5 % for the Series I and 23.1% and 11.9% for the Series II, respectively. The inhibitory activities produced by the Series I were generally higher than those by the Series II if the substituent(s) on the B-ring were the same. The Series I compounds with the OH group at the 3-position on the B-ring (**4**, **13**) had very low or no activity. The introduction of electron-withdrawing substituents such as Cl (**10**), CF₃ (**11**), and F (**15**, **16**) into the 4-position of the B-ring decreased inhibitory activities. In the case of Series II, the introduction of hydrophobic and electron-withdrawing substituents such as Cl (**31**, **56**, **58**), Br (**32**), I (**33**), and CF₃ (**34**) into the 3-position on the B-ring increased inhibitory activities.

Table 2-1. Inhibitory activities and classical QSAR parameters of dibenzoylhydrazines.

Number	Compound	IC ₅₀ (μM)	Inhibitory Activity (%) ^a	logit A ^b			log <i>P</i>	3- <i>F</i>	4- <i>F</i>	2- <i>L</i>
				Obsd.	Calcd.					
					Eq.2-2 ^c	Eq.2-3 ^d				
Series I										
1	B: H	100.4	17 ± 3.5	-0.69	-0.56	-	3.39	0	0	0
2	2-Me	79.9	22.3 ± 7.6	-0.54	-0.28	-	3.98	0	0	0.81
3	3-Me	48.2	36.8 ± 3.8	-0.23	-0.28	-	3.98	0.01	0	0
4	3-OH ^e	ND ^f	1 ± 0.2	-	-	-	3.34	0.33	0	0
5	3-OMe	58.1	34.2 ± 5.5	-0.28	-0.39	-	3.75	0.29	0	0
6	3OEt	39.9	50.9 ± 9.7	0.02	-0.14	-	4.28	0.26	0	0
7	4-Me	ND	30.9 ± 2.2	-0.35	-0.31	-	3.95	0	0.01	0
8	4-nPr	15.2	58.4 ± 11.3	0.15	0.11	-	4.86	0	0.01	0
9	4-iPr	ND	49 ± 12.3	-0.02	0.09	-	4.91	0	0.04	0
10	4-Cl	ND	14.2 ± 3.5	-0.78	-0.79	-	4.31	0	0.42	0
11	4-CF ₃ ^e	ND	0.9 ± 0.4	-	-	-	4.66	0	0.38	0
12	2,3-Me ₂	ND	41.1 ± 7.9	-0.16	-0.07	-	4.43	0.01	0	0.81
13	2-Me,3-OH ^e	ND	0.5 ± 1.5	-	-	-	3.84	0.33	0	0.81
14	2,3,4-Me ₃	ND	54.2 ± 5.4	0.07	0.12	-	4.88	0.01	0.01	0.81
15	2,3,4-F ₃ ^e	ND	2.5 ± 1.6	-	-	-	3.64	0.45	0.45	0.59
16	2,4,5-F ₃ ^e	ND	1.9 ± 0.4	-	-	-	3.71	0	0.45	0.59
17	tebufenozide (B:4-Et)	21.5	53.3 ± 15	0.06	-0.16	-	4.25	0	0	0
18	methoxyfenozide (B: 2-Me, 3-OMe)	44.3	32 ± 6.7	-0.33	-0.41	-	3.7	0.29	0	0.81
Series II										
19	B: H	ND	5.4 ± 1.9	-1.25	-	-1.36	2.59	0	0	0
20	2-F	ND	5.9 ± 0.8	-1.21	-	-1.24	2.63	0	0	0.59
21	2-Cl	ND	5.3 ± 0.2	-1.25	-	-1.01	2.75	0	0	1.46
22	2-Br	ND	10.1 ± 0.2	-0.95	-	-0.84	2.91	0	0	1.76
23	2-I	ND	21.8 ± 0.5	-0.55	-	-0.62	3.11	0	0	2.17
24	2-CF ₃	ND	15.6 ± 3.8	-0.73	-	-0.84	3.02	0	0	1.24
25	2-NO ₂ ^e	ND	1.7 ± 1.2	-	-	-	1.99	0	0	1.38
26	2-Ph	12.5	80.3 ± 4.9	0.61	-	0.21	3.77	0	0	4.22
27	2-OMe	ND	6.7 ± 1.3	-1.14	-	-1.23	2.37	0	0	1.92
28	2-SMe	ND	7 ± 3.3	-1.13	-	-0.82	2.84	0	0	2.24
29	3-Me	ND	11.5 ± 3.7	-0.89	-	-0.96	3.11	0.01	0	0
30	3-F	ND	7.6 ± 3.4	-1.08	-	-0.85	2.88	0.45	0	0
31	3-Cl	45.7	27.3 ± 2.9	-0.43	-	-0.4	3.49	0.42	0	0
32	3-Br	ND	33 ± 4.5	-0.31	-	-0.28	3.62	0.45	0	0

Table 2-1. Continued.

Number	Compound	IC ₅₀ (μM)	Inhibitory Activity (%) ^a	logit A ^b			log <i>P</i>	3- <i>F</i>	4- <i>F</i>	2- <i>L</i>
				Obsd.	Calcd.					
					Eq.2-2 ^c	Eq.2-3 ^d				
33	3-I	ND	63.9 ± 10.8	0.25	-	-0.11	3.87	0.42	0	0
34	3-CF ₃	43.8	33.4 ± 10.5	-0.3	-	-0.26	3.7	0.38	0	0
35	3-NO ₂	64.4	16.6 ± 5.3	-0.7	-	-0.84	2.73	0.65	0	0
36	3-CN	ND	9.5 ± 0.9	-0.98	-	-1.12	2.48	0.51	0	0
37	3-OMe	ND	10.8 ± 2.5	-0.92	-	-1.01	2.81	0.29	0	0
38	4-Me	ND	7.3 ± 2.9	-1.1	-	-0.93	3.15	0	0.01	0
39	4-Et	ND	23 ± 2.8	-0.52	-	-0.59	3.59	0	0	0
40	4-nPr	ND	48.7 ± 5.2	-0.02	-	-0.23	4.06	0	0.01	0
41	4-F	ND	4.8 ± 1.5	-1.3	-	-1.15	2.87	0	0.45	0
42	4-CF ₃	ND	16.2 ± 3.5	-0.71	-	-0.52	3.68	0	0.38	0
43	4-CN	ND	4.9 ± 1.4	-1.29	-	-1.48	2.44	0	0.51	0
44	4-OMe	ND	6.9 ± 2.8	-1.13	-	-1.19	2.82	0	0.29	0
45	4-COMe	ND	5.3 ± 2	-1.25	-	-1.49	2.42	0	0.33	0
46	2,3-Me ₂	ND	9.3 ± 2.9	-0.99	-	-0.6	3.4	0.01	0	0.81
47	2,3-Cl ₂	ND	45 ± 10.9	-0.09	-	0.03	3.75	0.42	0	1.46
48	2-Me,3-OMe	ND	12.2 ± 1.7	-0.86	-	-0.38	3.46	0.29	0	0.81
49	2,4-Cl ₂	ND	40.7 ± 7.3	-0.16	-	-0.27	3.71	0	0.42	1.46
50	2,5-Me ₂	73.6	26.8 ± 3	-0.44	-	-0.61	3.4	0	0	0.81
51	2,5-Cl ₂	46.7	44.2 ± 9.8	-0.1	-	-0.24	3.75	0	0	1.46
52	2,6-F ₂	ND	4.2 ± 1.7	-1.36	-	-1.45	2.35	0	0	0.59
53	2,6-Cl ₂	ND	5.8 ± 0.6	-1.21	-	-0.79	3.03	0	0	1.46
54	2-F,6-Cl	ND	6.9 ± 2.3	-1.13	-	-1.21	2.67	0	0	0.59
55	3,4-Me ₂	ND	20.8 ± 5.7	-0.58	-	-0.54	3.65	0.01	0.01	0
56	3,4-Cl ₂ ^e	ND	98.9 ± 11.5	-	-	-	4.25	0.42	0.42	0
57	3,5-Me ₂	ND	22.4 ± 4.3	-0.54	-	-0.53	3.67	0.01	0	0
58	3,5-Cl ₂ ^e	ND	97.2 ± 6	-	-	-	4.29	0.42	0	0

a; Inhibitory activities of test compounds (30 μM) against quinidine (1 μM) transport by P-gp were measured (Mean ± standard deviation, n = 3), b; Logit-transformed inhibitory activities, c; Calculated by Eq. 2-2, d; Calculated by Eq. 2-3, e; Excluded from a classical QSAR analysis, f; ND: not determined.

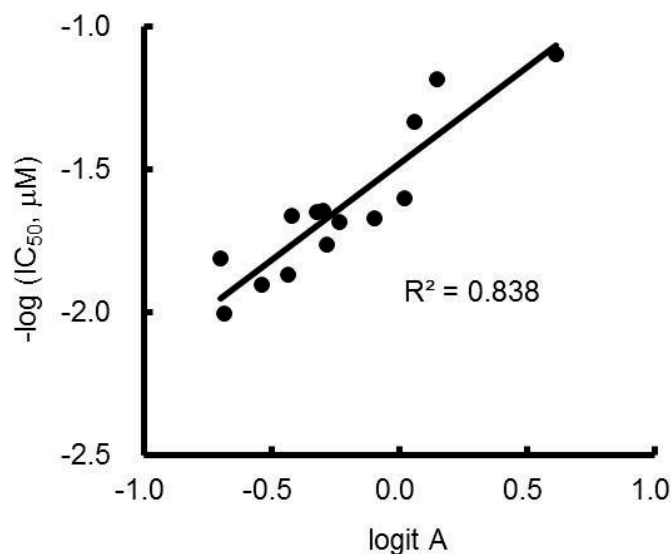


Figure 2-2. Relationship between IC₅₀ and logit A.

2-3-2. Classical QSAR analysis

The structure-inhibitory activity relationship was investigated using the classical QSAR analysis. The inhibitory activities produced by 13 compounds of Series I and 37 compounds of Series II of DBHs were analyzed separately. The log *P* term was introduced in QSAR equations because P-gp substrates were commonly lipophilic or amphiphilic, as described previously (Szerémy *et al.*, 2011; Wang *et al.*, 2003). Therefore, a good classical QSAR equation was obtained for each series.

Series I:

$$\text{logit A} = 0.463 (\pm 0.191) \log P - 1.58 (\pm 0.793) 4-F - 2.12 (\pm 0.807)$$

$$n = 13, s = 0.141, r^2 = 0.813, F_{2,10} = 21.7 \quad (\text{Eq. 2-2})$$

Series II:

$$\begin{aligned} \text{logit A} = & 0.770 (\pm 0.145) \log P + 0.645 (\pm 0.382) 3-F + 0.159 (\pm 0.080) 2-L \\ & - 3.36 (\pm 0.456) \end{aligned}$$

$$n = 37, s = 0.211, r^2 = 0.827, F_{3,33} = 52.4 \quad (\text{Eq. 2-3})$$

In these and subsequent equations, n is the number of compounds used in regression analyses, s is the standard deviation, r^2 is the coefficient of determination, and F is the F-statistics value. The values in parentheses are 95% confidence intervals of the regression coefficients and the intercept. The inhibitory activities of the Series I compounds were explained by $\log P$ and the field effect of the B-ring 4-substituent (4- F), whereas the activities of the Series II compounds were explained by $\log P$, the field effect of the B-ring 3-substituent (3- F), and the length of the B-Ring 2-substituent (2- L). The observed and calculated inhibitory activity values by Eqs. 2-2 and 2-3 as well as the physicochemical parameters of DBHs are listed in Table 2-1. Calculated versus observed activity values are shown in Figure 2-3. The inhibitory activities for the excluded compounds were calculated using Eq. 2-2 or Eq. 2-3 in order to examine the prediction ability of the obtained QSAR models. Their observed and calculated activity values are listed in Table 2-2.

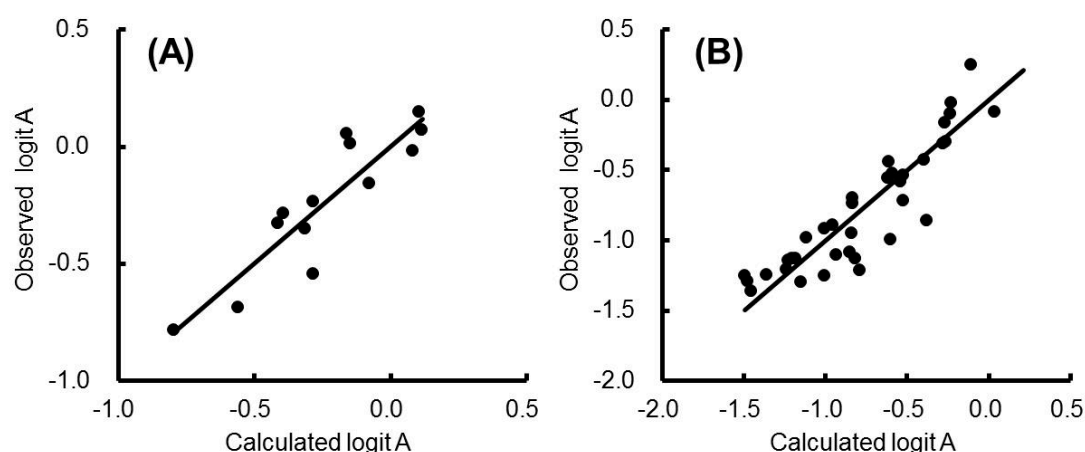


Figure 2-3. Observed versus calculated logit A, (A); Series I, (B); Series II.

Table 2-2. Calculated inhibitory activities of compounds excluded from a classical QSAR analysis.

QSAR analysis:				
Number	Compound	Inhibitory Activity (%)		
		Obsd.	Calcd.	
			Eq.2-2 ^a	Eq.2-3 ^b
Series I				
4	3-OH	1	20.9	-
11	4-CF ₃	0.9	21.3	-
13	2-Me,3-OH	0.5	31	-
15	2,3,4-F ₃	2.5	6.6	-
16	2,4,5-F ₃	1.9	7.1	-
Series II				
25	2-NO ₂	1.7	-	2.4
56	3,4-Cl ₂	98.9	-	60.5
58	3,5-Cl ₂	97.2	-	62.2

a; Calculated by Eq. 2-2, b; Calculated by Eq. 2-3.

2-3-3. CoMFA analysis

The structure-inhibitory activity relationship was investigated using a 3D-QSAR, CoMFA. The inhibitory activities of two series of DBHs were separately analyzed. For Series II, the compounds containing an iodine atom (**23**, **33**) were not included because their MNDO esp charges were not available. Since log *P* was the most significant parameter in the classical QSAR analysis, the log *P* term was introduced into CoMFA.

As a result, significant Eqs. 2-4 and 2-5 were obtained for the Series I and Series II compounds, respectively.

Series I:

$$\text{logit A} = -1.99 + 0.409 \log P + [\text{CoMFA field term}]$$

$$n = 13, s = 0.223, r^2 = 0.484, s_{cv} = 0.250, q^2 = 0.354, \text{CN} = 1$$

$$\text{Electronic } 2.3\%, \text{ Steric } 12.0\%, \log P \text{ } 85.7\% \quad (\text{Eq. 2-4})$$

Series II:

$$\text{logit A} = -3.21 + 0.777 \log P + [\text{CoMFA field term}]$$

$$n = 35, s = 0.255, r^2 = 0.712, s_{cv} = 0.277, q^2 = 0.661, \text{CN} = 1$$

$$\text{Electronic } 1.9\%, \text{ Steric } 4.2\%, \log P \text{ } 93.9\% \quad (\text{Eq. 2-5})$$

In these equations, s_{cv} and q^2 are the leave-one-out cross-validated standard error and correlation coefficient, respectively, CN is the number of latent variables. The relative contribution (%) of descriptors to the correlation equations was also shown.

Figure 2-4 (A) shows an overlay of the structure of compound (**8**), which has high activity among mono-substituted derivatives, with the major electrostatic and steric potential contour maps of Series I drawn according to Eq. 2-4. Figure 2-4 (B) shows an overlay of the structure of compound (**32**), which is a mono-substituted derivative having high activity, with the major electrostatic and steric potential contour maps of Series II drawn according to Eq. 2-5. The red areas in Figure 2-4 indicate regions where negative electrostatic interactions with the receptor increase the activity, whereas the blue areas show the reverse case. The green areas in Figure 2-4 indicate regions where the submolecular bulk is well accommodated with an increase in activity, whereas the yellow areas indicate regions where the submolecular bulk is unfavorable for activity.

According to Eqs. 2-4 and 2-5, hydrophobic factor, $\log P$ was most important for the activity in both series. Besides, electropositive and bulky 4-substituents of the B-ring

were favorable for the activity of the Series I. For the Series II, the electronegative and bulky 3-substituents, and electropositive 2-substituents of the B-ring were favorable for the activity.

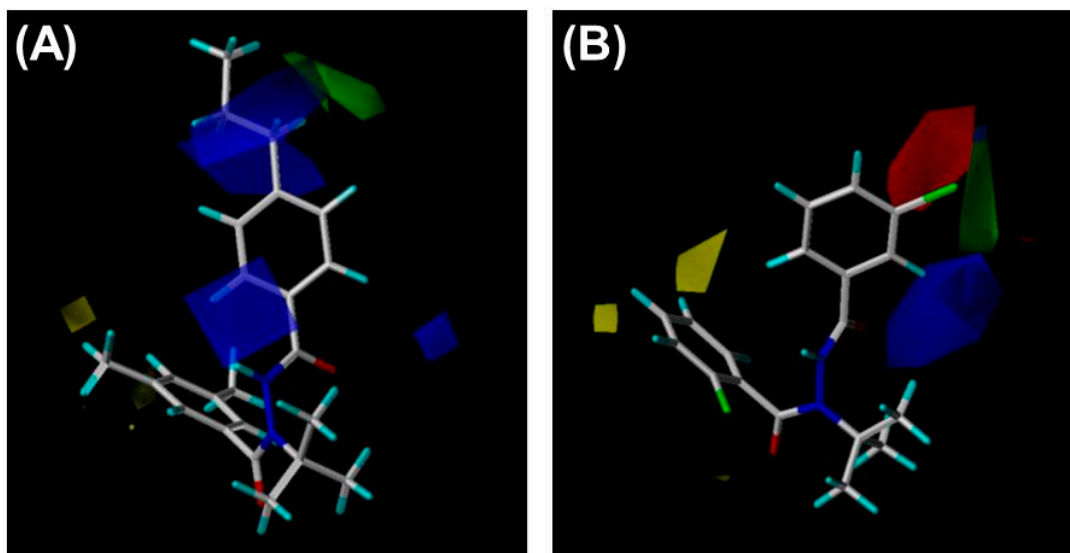


Figure 2-4. Overlay of the structure of compound (**8**) with the major CoMFA electrostatic and steric potential contour maps of Series I drawn according to Eq. 2-4 (A), and compound (**32**) with the major electrostatic and steric potential contour maps of Series II drawn according to Eq. 2-5 (B). Contours are shown to surround regions where a positive (blue) or negative (red) electrostatic potential increases the activity, and increased steric bulk increases (green) or decreases (yellow) the activity.

2-4. Discussion

Previous studies demonstrated that the binding site of P-gp is in the transmembrane domain (Tang *et al.*, 2004; Martin *et al.*, 2000; Loo and Clarke, 2002; Aller *et al.*, 2009; Szewczyk *et al.*, 2015; Li *et al.*, 2014). Aller *et al.* (2009) and Szewczyk *et al.* (2015) showed that cyclic peptides (P-gp substrates) bind within the transmembrane domain using the X-ray crystal structure of the complex of mouse P-gp in the inward-facing conformation with these peptides (Aller *et al.*, 2009; Szewczyk *et al.*, 2015; Li *et al.*, 2014). Several studies have also reported that drug partition into a lipid bilayer is important for the drug-P-gp interaction (Sharom, 2014; Seelig and Landwojtowicz, 2000; Romsicki and Sharom, 1999; Higgins and Gottesman, 1992). Romsicki *et al.* (1999) found that drug binding affinity increased as the drug partitions in lipids (drug concentration within the membrane) increased in a binding study using P-gp reconstituted in three different lipid compositions. Several studies have shown that the $\log P$ of transport substrates is important for their interaction with P-gp (Wang *et al.*, 2003; Szerémy *et al.*, 2011), indicating the partition of substrates into the membrane increases their excretion rate by P-gp.

In a classical QSAR analysis of inhibitory activity, the coefficients of the $\log P$ term in both series were positive (Eqs. 2-2 and 2-3). Moreover, 44.5% and 71.3% of the total variation in inhibitory activities for the Series I and Series II, respectively, were explained by their $\log P$, as shown in Eqs. 2-6 and 2-7.

Series I:

$$\log A = 0.415 (\pm 0.308) \log P - 1.98 (\pm 1.30)$$

$$n = 13, s = 0.232, r^2 = 0.445, F_{1,11} = 8.82 \quad (\text{Eq. 2-6})$$

Series II:

$$\log A = 0.818 (\pm 0.178) \log P - 0.333 (\pm 0.568)$$

$$n = 37, s = 0.264, r^2 = 0.713, F_{1,35} = 86.9 \quad (\text{Eq. 2-7})$$

Thus, $\log P$ was the most important parameter for determining inhibitory activity, indicating that the partition of DBHs into the membrane is important for the interaction with P-gp; however, the hydrophobicity of compounds may also directly contribute to their binding to P-gp. The higher activities of Series I compounds than those of Series II may be due to the higher $\log P$ of the former compounds (mean value: 4.10 for the Series I versus 3.18 for the Series II). In the classical QSAR analysis, if ligands interact with the receptor protein buried in biological membranes, the coefficient of the $\log P$ term is expected to be approximately 1.0 (Hansch and Leo, 1995). On the other hand, it is expected to be approximately 0.5 if ligands interact with the receptor protein on the surface area of biological membranes (Hansch and Leo, 1995). The coefficients of the $\log P$ term in Eq. 2-3 (0.770) and Eq. 2-7 (0.818) for the Series II were close to 1.0, suggesting that this series of compounds interact with P-gp buried in the transmembrane domains. However, the coefficients of the $\log P$ term in Eq. 2-2 (0.463) and Eq. 2-6 (0.415) for the Series I were close to 0.5. Since it is unlikely that the binding sites of these compounds exist on the surface area of the membranes, this is considered to reflect a limitation in the structural variety due to the small number of the Series I compounds.

The effects, except for $\log P$, on inhibitory activity were different between Eqs. 2-2 and 2-3. These significant differences in the substituent effects of the B-ring in each series suggest the different binding modes of the compounds to P-gp. The inhibitory activities of the Series I was explained by the field effect of the B-ring 4-substituent, $4-F$ (Eq. 2-2). The negative contribution of $4-F$ indicates that the inductive electron-donating 4-substituents on the B-ring increase the interaction of DBHs with P-gp. This inductive electron-donating effect may be important for interactions with electronegative residues in P-gp or may influence the electron density of the 1-carbonyl oxygen atom of the DBH skeletal chain. If the latter assumption is correct, the Swain-Lupton resonance constant (R), which represents the resonance effects of 4-substituents as well as their inductive effects, may be significant. However, the introduction of the parameter R did not give a significant equation. Therefore, the inductive electron-donating 4-substituents on the B-ring are considered to interact with electronegative residues in P-gp. On the other hand, the inhibitory activities of the Series II were explained by the positive contribution of $3-F$, and the introduction of the resonance constant R of the B-ring substituents was insignificant (Eq. 2-3). These results indicate that electrostatic interactions between the electronegative substituents at the 3-positions of the B-ring, and electropositive residues in P-gp are important for inhibitory activity. Regarding the Series II, the $2-L$ term with a positive coefficient was also significant, showing that the longer 2-substituents on the B-ring increased inhibitory activity. A certain space was found around the 2-position of the B-ring in P-gp, as discussed in Chapter 3.

Five and three compounds were excluded for the Series I and Series II from the QSAR analysis, respectively, due to possible logit-transformation errors. The activities

of the excluded compounds were calculated using Eq. 2-2 or Eq. 2-3 (Table 2-2). The activities of compounds **15**, **16**, and **25** were predicted well, and the calculated activities of compounds **56** and **58** were acceptable. However, the activities of compounds **4**, **11**, and **13** were predicted to be higher than those observed. Compounds **4** and **13** have a hydroxyl group, which may function as a hydrogen-bond donor and/or acceptor. P-gp contains a high fraction of side chains with a hydrogen-bonding characteristic in transmembrane domains (Seelig, 1998). The lower inhibitory activities than the calculated values of these compounds may be due to their hydrogen-bonding interactions with P-gp, leading to an irrelevant binding mode. Previous studies reported that the hydrogen bonding of compounds influences the binding activities of acetylcholinesterase inhibitors or insecticidal activities of DBHs (Fujita *et al.*, 1977; Nakagawa *et al.*, 1999). In these reports the indicator variable which takes one for hydrogen-bonding substituents and 0 for the others of ligands was used in QSAR equations. In a QSAR analysis, 5-6 compounds are required in order to introduce one independent variable into the QSAR equation, thereby avoiding chance correlations (Topliss and Costello, 1972). Since only 2 compounds had a hydroxyl group in this study, the indicator variable for hydrogen bonding was not applicable. More compounds with a hydroxyl group at the 3-position of the B-ring are needed in order to evaluate the effects of hydrogen bonding on inhibitory activity. The observed inhibitory activity of compound **11** having 4-CF₃ was also lower than the calculated value. The CF₃ group exerts electron-withdrawing effects, which decrease inhibitory activity, but is fairly hydrophobic. Since the backbone structure is the same in each series, variation of log *P* values represents the hydrophobicity of the substituents in each compound set used. The hydrophobicity of the CF₃ group may be over-estimated in the calculated value.

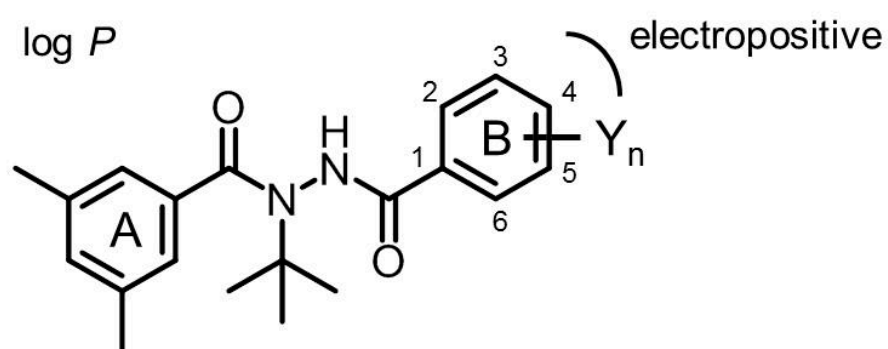
The author also developed CoMFA models for the two series of DBHs, separately. In the CoMFA equations, $\log P$ was most important for the activity in the both series, being consistent with the classical QSAR results. In the CoMFA result for Series I, electropositive and bulky 4-substituents of the B-ring were favorable for the activity (Figure 2-4 (A)), whereas in classical QSAR Eq. 2-2, the only electropositive effect of 4-substituents was significant. A positive correlation between steric effects of the 4-substituent (4-*L*) and $\log P$ was found in the mono-substituted compounds having a 4-substituent on the B-ring ($r^2 = 0.64$, data not shown). This collinearly seems to cause the difference between classical QSAR and CoMFA results. In the CoMFA result for Series II, electronegative and bulky 3-substituents of the B-ring were favorable for the activity (Figure 2-4 (B)), while in classical QSAR Eq. 2-3, the only electronegative effect of 3-substituents was significant. Although the favorable regions around the 3-position in CoMFA is due to the compounds with electronegative and bulky substituents such as halogen and CF_3 at the position on the B-ring, which have high activities, it is difficult to explain the difference of the results between these two analyzes. Regarding the effect of 2-substituents, electropositive substituents were favorable in CoMFA whereas long substituents (2-*L*) were favorable in the classical QSAR analysis. A negative correlation between 2-*L* and 2-*F* was found in the mono-substituted compounds having a 2-substituent of the B-ring ($r^2 = 0.54$, data not shown). It is considered that this collinearly caused the difference between two analyses.

The coefficient of determination of CoMFA models (r^2 : 0.484 and 0.712 for Series I and Series II, respectively) was lower than those of the classical QSAR equations (r^2 : 0.813 and 0.827 for Series I and Series II, respectively). CoMFA was originally developed in order to evaluate steric and electrostatic effects in ligand-receptor

interaction. The classical QSAR may be better than CoMFA for analyses of activity which hydrophobic effects of compounds mainly control. The findings that electrostatic and steric effects of B-ring substituents were different between Series I and Series II suggested the difference of the binding mode of two series of derivatives to P-gp. The classical QSAR results were summarized in Figure 2-5.

In a previous study (Kanaoka *et al.*, 2013), the human P-gp ATPase activities of the same series of DBHs used in this study were evaluated, and 3D-QSAR analysis for the ATPase activities was conducted. The substituent effects in QSAR equations for the inhibitory activities were different from those for the ATPase activities because there was no correlation between two activities ($r^2 = 0.0982$, data not shown). Several previous studies have reported that there is a stronger correlation of *in vivo* P-gp function at BBB with the results of the *in vitro* transport study using cells than with the ATPase activities as described in Chapter 1 (Adachi *et al.*, 2001; Feng *et al.*, 2008). Thus, it is considered that the *in vitro* transport study using cells is more useful assay system to predict the *in vivo* P-gp function. Although the reasons for the differences among these *in vitro* P-gp assay systems have not been elucidated yet, the most critical step to stimulate ATPase activity might be different from the initial binding of ligands to P-gp.

(A)



(B)

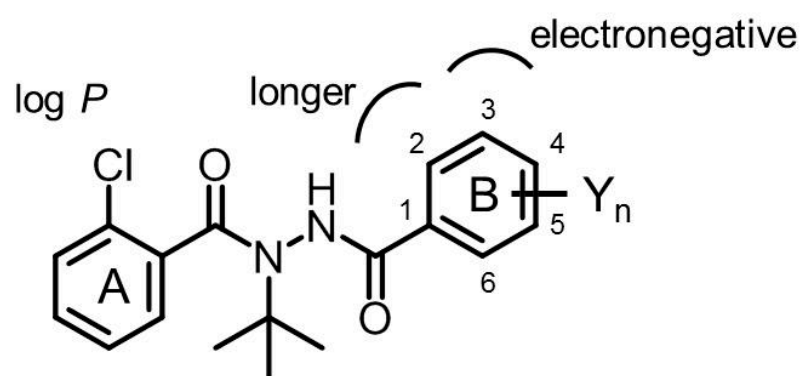


Figure 2-5. Summary of classical QSAR results, (A); Series I, (B); Series II.

CHAPTER 3:

Molecular docking of dibenzoylhydrazines to human P-glycoprotein

3-1. Introduction

In the computational approaches in order to design drugs and compounds, there are two types of approaches; ligand-based and structure-based approaches. In the ligand-based approach, including QSAR analysis, the compound optimization was conducted based on the structure of the ligand and 3D structure of the target protein are not used. On the other hand, in the structure-based approach, the compound optimization was conducted based on the structure of the receptor and ligand. Molecular docking study, one of the structure-based approaches, is a computational method to find the interaction and binding mode between protein and ligand.

A large number of studies have been conducted in order to identify the substrate-binding sites of P-gp. P-gp is known to contain at least two binding sites (Shapiro and Ling, 1997); the H-Site at which Hoechst 33342 and colchicine bind, and the R-site at which rhodamine 123 and anthracyclines bind (Shapiro and Ling, 1997). A third binding site for prazosin and progesterone has also been reported (Shapiro *et al.*, 1999). Therefore, P-gp appears to contain multiple binding sites within a cavity located at the interface between transmembrane domains (Tang *et al.*, 2004; Martin *et al.*, 2000; Loo and Clarke, 2002).

After Aller *et al.* reported the first mammalian (mouse) structure of P-gp, several molecular docking studies were conducted in order to investigate the mechanisms underlying P-gp substrate/inhibitor recognition (Chen L *et al.*, 2012). However, few studies have predicted P-gp substrates/inhibitors using the both QSAR and modeling methods (Pajeva *et al.*, 2009). Then, a docking simulation study on DBHs exhibiting high inhibitory activities was attempted in order to propose their binding sites and modes.

3-2. Materials and Methods

3-2-1. Modeling of human P-glycoprotein

Modeling of human P-gp was performed using the homology modeling software PDFAMS pro ver. 2.0 (Protein Discovery Full Automatic Modeling System; In-Silico Sciences, Inc.; Tokyo, Japan) and SYBYL ver. 7.3. Szewczyk *et al.* previously reported four nucleotide-free structures for mouse P-gp in an inward-facing conformation that were complexes with cyclic peptides such as QZ-Ala (PDB code: 4Q9I), QZ-Val (4Q9J), QZ-Leu (4Q9K), and QZ-Phe (4Q9L) (Szewczyk *et al.*, 2015). The co-crystal structure with QZ-Ala was used as a template protein for human P-gp modeling because QZ-Ala exhibited the highest P-gp ATPase activity among them and induced the largest conformational change in TM4 in P-gp which was described in 4.4 (Szewczyk *et al.*, 2015). The primary sequence of human P-gp and its alignment with mouse P-gp by Aller *et al.* (2009) (Supplementary Material, Fig. S1 of Aller *et al.* (2009)) were used (sequence alignment similarity 93%) in this study. The P-gp models constructed were energy minimized for 5,000 iterations of conjugated gradients using the force field and partial charges of the molecular mechanics MMFF94. The coordinates of backbone

atoms were fixed during energy minimization.

3-2-2. Docking simulation of DBHs to P-gp

GOLD ver. 5.1 (Cambridge Crystallographic Data Centre (CCDC) Software Ltd., Cambridge, UK) was used to dock the compounds: tebufenozide (**17**), B-ring: 3-OEt (**6**), and 4-*n*Pr (**8**) for Series I, and B-ring: 3-I (**33**) and 4-*n*Pr (**40**) for Series II, which exhibit high inhibitory activities among mono-substituted derivatives. The structures in CoMFA described in Chapter 2 were used as the initial structures of these DBHs. All residues within 15.0 Å of F239 in the cavity of the inner leaflet of the membranes were selected for the docking site in order to cover the whole hydrophilic cavity of the inner leaflet of the membranes. A cavity detection algorithm was used for selected atoms in order to restrict the region of the binding-site cavity. A Genetic Algorithm (GA) was applied for GOLD docking and automatic GA settings were used for all calculations. Operator weights for the crossover, mutation, migration (95, 95, and 10, respectively), hydrogen bonding (2.5 Å), and van der Waals (4.0 Å) parameters were set as default values throughout the docking.

3-3. Results

The homology model of human P-gp was shown in Figure 3-1 (A). The cavity surface areas of P-gp were also rendered with hydrophobic potential (brown; hydrophobic, green; neutral, light blue; hydrophilic) in Figure 3-1 (A). There were hydrophobic regions in the internal cavity, and hydrophilic regions were observed in the cavity of the inner leaflet of the membranes. The docking site (all residues within 15.0 Å of F239 in the cavity of the inner leaflet of the membranes) was shown in Figure 3-1 (B). Docked poses were ranked based on the ChemPLP score, which represents the sum

of receptor–ligand hydrogen bonding, van der Waals, torsional, and hydrophobic interaction energies. Higher ChemPLP scores indicate better binding interactions between the compounds and receptors. Three, three, and five poses were obtained for tebufenozide (B-ring: 4-Et, (**17**)), and B-ring: 3-OEt (**6**) and 4-nPr (**8**) of the Series I exhibiting high inhibitory activities, respectively. The poses between these three compounds were compared. As a result, a common pose was found and, thus, was assumed to show the binding conformations of the Series I. Ten and ten poses were obtained for B-ring: 3-I (**33**) and 4-nPr (**40**) of the Series II, respectively. A common pose was also found for these two compounds. The conformation and orientation of the common pose of the Series II was similar to that of the Series I, but was rotated slightly counterclockwise. The overlay of the assumed binding poses of compounds **8** and **33** docked into the homology model of human P-gp were drawn in Figures 3-2 and 3-3.

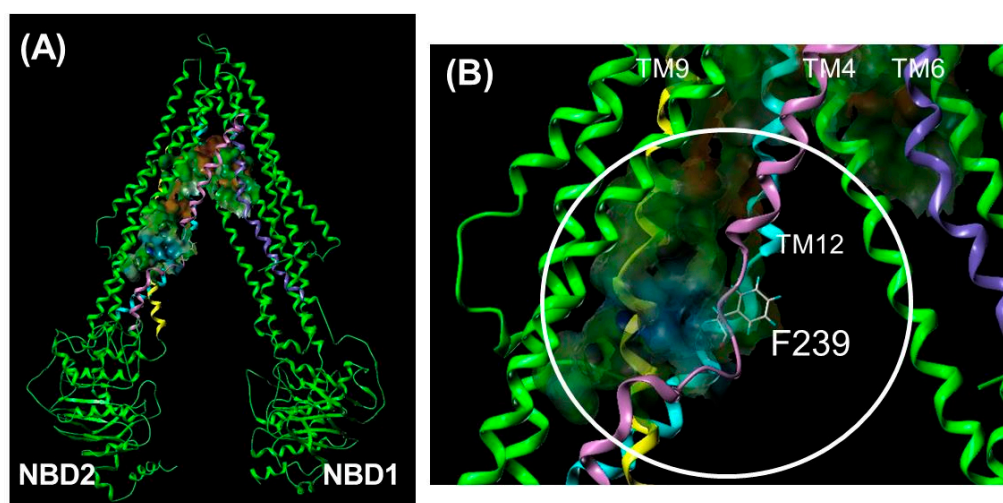


Figure 3-1. (A) Homology model of human P-gp. The mouse P-gp structure (PDB code: 4Q9I) was used as a template. The cavity surface areas of P-gp were rendered with hydrophobic potential (brown; hydrophobic, green; neutral, light blue; hydrophilic). (B) The docking site on the inner leaflet membranes of P-gp in this study (white circle). Nucleotide binding domains (NBD1, NBD2) and transmembrane domains (TM4, TM6, TM9, TM12) are labeled. TM4, TM6, TM9, and TM12 are shown in pink, wisteria, yellow, and cyan, respectively.

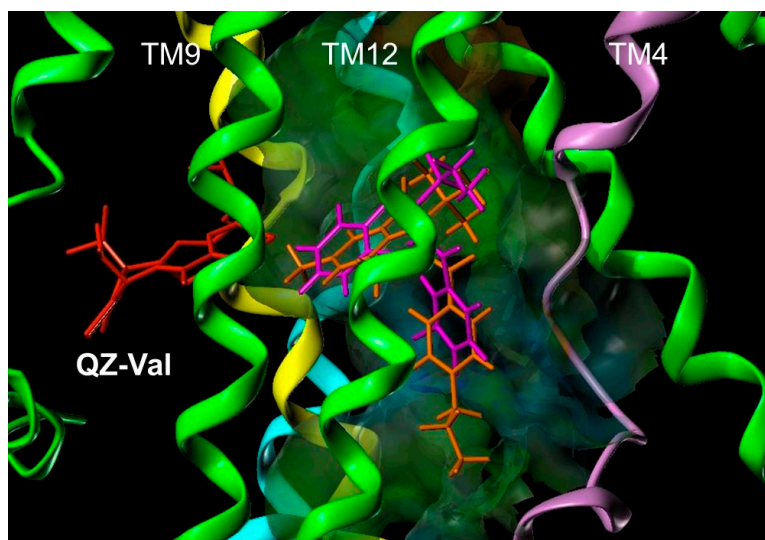


Figure 3-2. Overlay of the assumed binding poses of compound (8) (orange) of Series I and compound (33) (magenta) of Series II docked into the homology model of human P-gp. QZ-Val (red orange) in the complex structure of mouse P-gp (PDB code: 4Q9J, ref 19) was shown in the human P-gp model. TM4, TM9, and TM12 are shown in pink, yellow, and cyan, respectively.

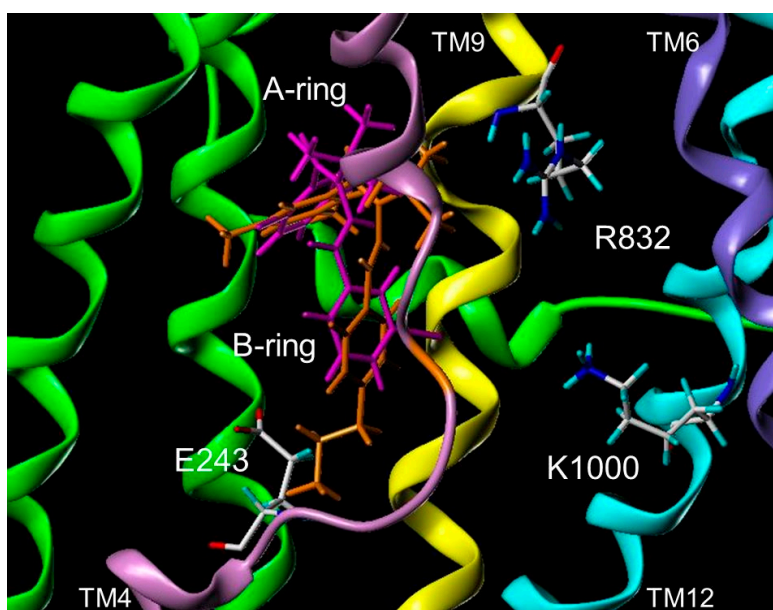


Figure 3-3. Poses of compound (8) (orange) of Series I and compound (33) (magenta) of Series II with the amino acid residues of P-gp, which appear to play important roles in protein-ligand interactions. Compounds were docked into the cavity of the inner leaflet membranes of human P-gp homology model. TM4, TM6, TM9, and TM12 are shown in pink, wisteria, yellow, and cyan, respectively.

3-4. Discussions

In 2009, Aller *et al.* reported the structure of mouse P-gp with cyclic peptides such as QZ-Ala (Aller *et al.*, 2009; Li *et al.*, 2014), and revealed that the ligands bind to the internal central cavity. Previous molecular docking studies (Chen *et al.*, 2012; Pajeva *et al.*, 2009) have been conducted based on the structure of mouse P-gp, and ligands were docked into the internal central cavity surrounded by the hydrophobic residues of P-gp because it was the common binding site of ligands in complex crystal structures. Then, the author initially docked DBHs into the internal central cavity of P-gp. However, it was difficult to explain the results of the QSAR analysis based on the docking models obtained. Szewczyk *et al.* found an additional binding site on the surface of P-gp in the inner leaflet of the membrane, which is the putative entry site of substrates, using a co-crystal structure of mouse P-gp with a cyclic peptide (QZ-Val) (Aller *et al.*, 2009). Moreover, they reported that the structure of TM4 in *apo* P-gp was slightly different from that of cyclic peptide-bounded P-gp. The binding of the ligand to P-gp induced a conformational change in TM4 in P-gp, and TM4 and TM6 were reported to comprise an intramembrane portal for substrate entry (Aller *et al.*, 2009). Therefore, the hydrophilic cavity of the inner leaflets of membranes was considered to be important as the site for ligand-P-gp interactions as well as the hydrophobic internal central cavity. Thus, DBHs were docked into the hydrophilic cavity of the inner leaflet of the membranes (Figure 3-1(B)). As shown in Figure 3-2, both series of compounds were docked into the same binding site; however, the binding mode was slightly different in each series. QZ-Val in the complex structure of mouse P-gp (PDB code: 4Q9J) was also shown in the human P-gp model (Figure 3-2). The binding site of the DBHs was close to that of QZ-Val, which just attached to the surface of P-gp. In the Series I, the acidic

residue E243 was found near the 4-substituents of the B-ring (around 4 Å, Figure 3-3). In the Series II, the 3-substituents of the B-ring were surrounded by the basic residues, R832 and K1000 (around 5-6 Å, Figure 3-3). These findings support the classical QSAR results. The author also docked compound **26** having 2-Ph on the B ring, which exhibited the highest inhibitory activity among the mono-substituted Series II, into the same site. The poses obtained were different from those of the two compounds (**33**, **40**) because the binding site was slightly too small to accommodate the 2-Ph substituent. However, a certain space was found around the 2-position of the B-ring. When compound **26** binds to P-gp, a small conformational change similar to the induced fit in P-gp may occur in order to accommodate the compound.

Based on the results of the present study, the cavity of the inner leaflets of membranes appears to be the initial binding site of DBHs in P-gp. Szewczyk *et al.* (2015) proposed that ligands firstly bind in the lower-affinity site near the inner leaflet of the membranes, and then move to the higher-affinity site within the central binding cavity. According to this proposal, DBHs may initially interact electrically with P-gp at the docking site in the inner leaflets of the membranes, as represented by the electronic terms in Eqs. 2-2 and 2-3, and then move to the central cavity by a hydrophobic interaction with P-gp. The hydrophobicities of DBHs are important for the hydrophobic interaction in the central cavity as well as the partition of ligands into the membrane.

Summary and Conclusions

Summary:

Chapter 1

In order to evaluate the interactions of DBHs with other chemicals in humans, it was determined whether DBHs are P-gp transport substrates using both the *in vitro* bidirectional transport assay and the *in vivo* study of rats. In the *in vivo* study, the influence of P-gp inhibitors on the brain to plasma ratio of methoxyfenozide in rats was investigated. It was also examined the inhibitory effects of DBHs on quinidine transport by P-gp in order to ascertain whether these derivatives are inhibitors of P-gp. Based on the results, DBHs were concluded to be weak P-gp transport substrates and moderate P-gp inhibitors. Moreover, it was concluded that the risk of DBHs caused by interaction with other chemicals including drugs may be low by considering the DBHs' potential as the substrates and inhibitors of P-gp as well as their plasma concentrations as long as they are properly used. These kinds of studies are important for the risk assessments of agrochemicals. The risk related to the pharmacokinetic interactions of an agrochemical with other chemicals should be evaluated based on the plasma concentration of the agrochemical if it is a P-gp substrate and/or an inhibitor.

Chapter 2

The author evaluated the inhibition of P-gp-mediated quinidine transport by two series of DBHs (18 compounds having 3,5-Me₂ group and 40 compounds having 2-Cl group on the A-ring) using the *in vitro* bidirectional transport assay, and performed a

classical QSAR and CoMFA analyses in order to elucidate the mechanisms underlying P-gp substrate/inhibitor recognition. The results of the QSAR analysis identified the hydrophobic factor as the most important for inhibitory activities, while electronic and steric effects also influenced the activities. In the Series I, the electropositive 4-substituents on the B-ring are considered to interact with electronegative residues in P-gp. In the Series II, the electrostatic interactions between the electronegative substituents at the 3-positions of the B-ring and electropositive residues in P-gp are important for inhibitory activity. Regarding the 2-positions on the B-ring in the Series II, the longer substituents increased inhibitory activity. Therefore, there might be a certain space around the 2-position of the B-ring in P-gp. The different substituent effects observed in each series suggested the different binding modes of each series of DBHs

Chapter 3

The molecular docking study was conducted in order to propose binding sites and modes of two series of DBHs in P-gp. Two series of DBHs were docked into the hydrophilic cavity of the inner leaflets of membranes of P-gp; however, the binding mode was slightly different in each series. In the Series I, the acidic residue E243 of P-gp was found near the 4-substituents of the B-ring while in the Series II, the 3-substituents of the B-ring were surrounded by the basic residues, R832 and K1000 of P-gp. These findings supported the QSAR results.

Conclusion:

Based on the present study, the most important step for DBHs to interact with P-gp seems to be the partition into a lipid bilayer based on the results of QSAR analysis. Then, DBHs initially interact electrically with P-gp at the binding site in the inner

leaflets of the membranes in the inward-facing conformation, as represented by the docking study, and move to the central cavity by a hydrophobic interaction with P-gp. Thus the hydrophobicity of DBHs is also considered to be important for the hydrophobic interaction in the central cavity. This binding of DBHs triggers a structural change to the outward-facing conformation of P-gp. This structural conversion requires the hydrolysis of ATP. DBHs are then excluded as a result of decreased binding affinity. The proposed recognition mechanism of P-gp substrates was shown in Figure. The inhibitory effect of DBHs on quinidine transport may appear at the substrate entry site in the inner leaflets of the membranes in the inward-facing conformation.

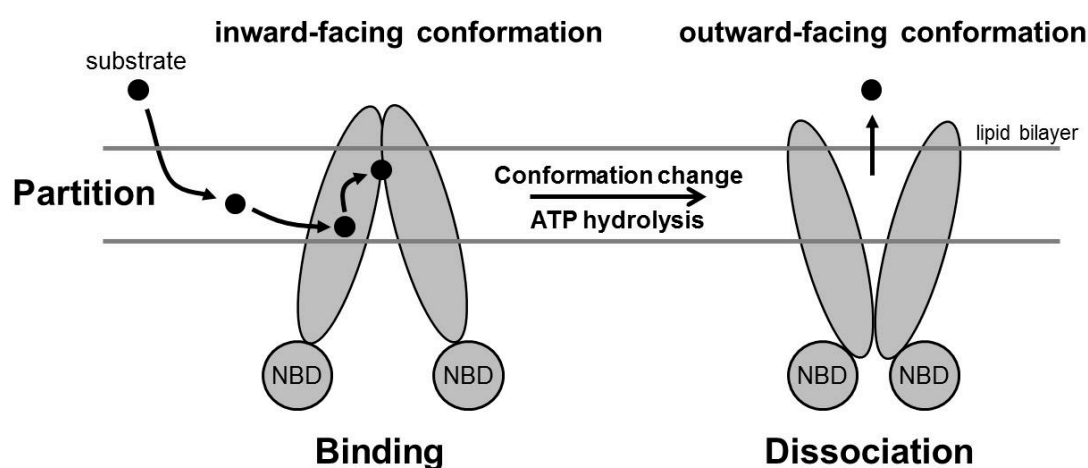


Figure. Proposed recognition mechanism of P-gp substrates.

Hydrophobic compounds easily partition into membranes and are also favorable for the hydrophobic interaction with P-gp. P-gp contributes to the elimination of diverse xenobiotics and drugs as an efflux pump. Compounds with weaker interactions with P-gp are preferred in order to develop drugs that target the CNS, anticancer drugs, or drugs that avoid DDI. The high hydrophobicity of compounds may increase their toxicities, while low hydrophobicity may decrease their efficacies as drugs. Therefore, the best approach to change the binding affinities of compounds to P-gp without influencing their toxicities or efficacies is to change their interactions with the pharmacophore of P-gp while maintaining appropriate hydrophobicity. The QSAR analysis represents a good approach to study the pharmacophore of protein receptors and enzymes. In this study, the author developed QSAR-based pharmacophore models of P-gp. These models will provide insights into the development of drugs and contribute to the elucidation of multidrug resistance mechanisms.

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Papers

Original Papers

Miyata, K., Nakagawa, Y., Kimura, Y., Ueda, K., Akamatsu, M. 2016. *In vitro* and *in vivo* evaluations of the P-glycoprotein-mediated efflux of dibenzoylhydrazines. *Toxicol. Appl. Pharmacol.* **298**, 40-47.

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