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High-Speed Screening and QSAR Analysis of Human ATP-Binding Cassette Transporter ABCB11 (Bile Salt Export Pump) To Predict Drug-Induced Intrahepatic Cholestasis

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Abstract: Human ATP-binding cassette transporter ABCB11 (SPGP/BSEP) mediates the elimination of bile salts from liver cells and thereby plays a critical role in the generation of bile flow. In the present study, we have developed in vitro high-speed screening and quantitative structure−activity relationship (QSAR) analysis methods to investigate the interaction of ABCB11 with a variety of drugs. Plasma membrane vesicles prepared from insect cells overexpressing human ABCB11 were used to measure the ATP-dependent transport of [^{14}C]taurocholate. Over 40 different drugs and natural compounds were tested to evaluate their interaction with ABCB11-mediated taurocholate transport. On the basis of the extent of inhibition, we have analyzed the QSAR to identify one set of chemical fragmentation codes closely associated with the inhibition of ABCB11. This approach can be used to predict compounds with a potential risk of drug-induced intrahepatic cholestasis.

Keywords: ABC transporter; ABCB11; bile salt; intrahepatic cholestasis; troglitazone

Introduction

In the last decade of the 20th century, the development of high-throughput screening and combinatorial chemistry technologies helped accelerate the drug discovery process. In the 21st century, emerging genomic technologies (i.e., bioinformatics, system biology, functional genomics, and pharmacogenomics) are shifting the paradigm for drug discovery and development. Nevertheless, drug discovery and development remain high-risk and high-stakes ventures with long and costly time lines.[1−3] The attrition of drug candidates in preclinical and development stages is a major problem in drug design. In at least 30% of the cases, this attrition is due to poor pharmacokinetics and toxicity. Since drug

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development costs have exponentially increased during the past twenty years, pharmaceutical companies have begun to seriously reevaluate their current strategies of drug discovery and development.[1−3]

In that light, we propose that transport mechanism based design might help to create new, pharmacokinetically advantageous drugs, and as such it should be considered an important component of drug design strategy.[4,5] Drug transporters, as well as drug-metabolizing enzymes, are important factors because they play pivotal roles in determining the pharmacokinetic profiles of drugs and, by extension, their overall pharmacological or adverse effects. For the prediction of drug-induced intrahepatic cholestasis in the drug discovery process, we have developed in vitro high-speed screening and quantitative structure−activity relationship (QSAR) analysis methods to investigate the interaction of the human bile salt export pump (BSEP/ABCB11) with drugs.

In 1995, the bile salt export pump was originally discovered as a sister gene of P-glycoprotein (spgp) in pig,[6] although its function was not clear at that time. Successive studies revealed that spgp transports various bile salts[7] and that disruption of the *spgp* gene in mice caused persistent intrahepatic cholestasis.[8] In humans, the bile salt export pump is encoded by the *ABCB11* gene, which belongs to the human ATP-binding cassette (ABC) transporter gene family.[9] Human ABCB11 is a 170 kDa glycoprotein that consists of 1321 amino acid residues and plays a major role in the hepatobiliary excretion of bile salts.[10] Several mutations in the *ABCB11* gene located on chromosome 2q24 have been reported to be associated with progressive familial intrahepatic cholestasis (PFIC) type II.[11,12] The vectorial transport of bile salts from blood into bile is essential for the generation of bile flow, solubilization of cholesterol in bile, and emulsification of lipids in the intestine.[13−15] The bile salts are the predominant form of glycine- or taurine-conjugated bile acids that are negatively charged at physiological pH. Under normal conditions, conjugated bile salts are excreted into bile via ABCB11 with the rank order of taurochenodeoxycholate > taurocholate > tauroursodeoxycholate > glycocholate.[16]

While the pathophysiological aspects of ABCB11 in liver diseases have been extensively studied, its application to drug discovery and development is still limited. Inhibition of the ABCB11 function by a drug or its metabolite(s) can result in hepatotoxicity caused by intrahepatic cholestasis. For example, inhibition of ABCB11 by troglitazone sulfate has been reported as a possible factor contributing to troglitazone-induced hepatotoxicity.[17,18] As a matter of fact, drug-induced hepatotoxicity is one of the major problems in drug development and postmarketing strategies.[19] Therefore, the selection of a candidate compound for preclinical studies and/or lead optimization in the drug discovery process is a critical step that can determine the speed and expenditure of clinical development. The present study provides a new strategy for

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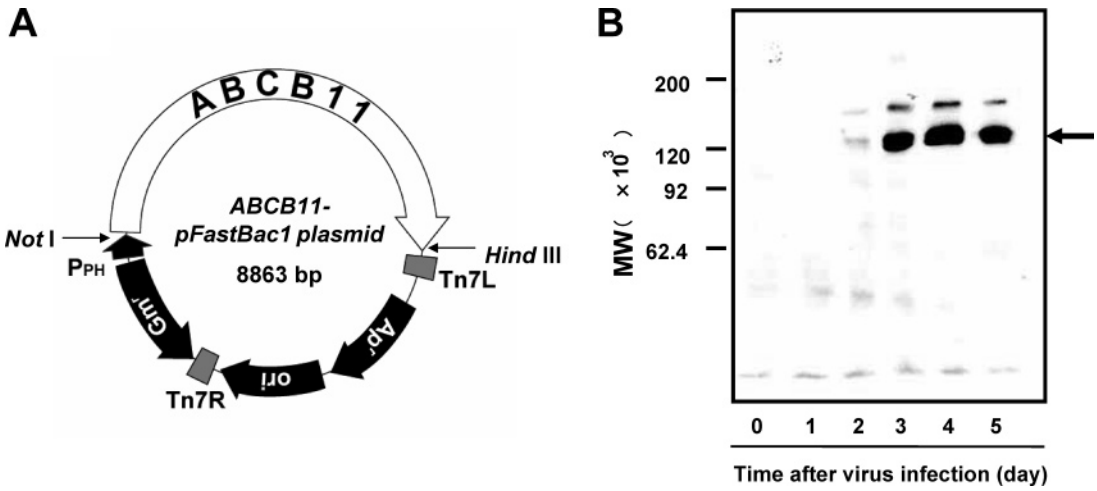

Figure 1. (A) Human ABCB11 cDNA inserted into the pFastBac1 vector. (B) Immunoblot analysis of ABCB11 expressed in Sf9 cells after baculovirus infection. Sf9 cells were infected with ABCB11-recombinant baculovirus on day 0 and then incubated for the different numbers of days indicated. Expression of ABCB11 in those cells was detected by immunoblotting as described in the Experimental Section. The ABCB11 protein is indicated by an arrow.

analyzing ABCB11−drug interactions. This strategy is considered to be practical and useful for selecting safe drug candidates.

Experimental Section

Chemicals and Biochemicals. The following compounds and therapeutic drugs were purchased from the commercial sources indicated in parentheses: ATP, epinephrine, norepinephrine, γ-aminobutyric acid (GABA), serotonin, melatonin, nifedipine, bepridil, fendiline, prenylamine, nicardipine, dexamethasone, prednisolone, cortisone, pinacidil, acetylsalicylic acid, indomethacin, acemetacin, ibuprofen, naproxen, mepirizole, vinblastine, etoposide, doxorubicin, daunorubicin, paclitaxel, 5-fluorouracil, quinidine, *p*-aminohippuric acid, penicillin G, novobiocin, and troglitazone (Sigma-Aldrich Co., St. Louis, MO); creatine kinase, ATP disodium salt, glutamic acid, dopamine, histamine, verapamil, diltiazem, betamethasone, nicorandil, actinomycin D, mitoxantrone, prazosin, methotrexate, glibenclamide, rapamycin, and rifampicin (Wako Pure Chemical Industries, Ltd., Osaka, Japan); glycine, Tris, HEPES, EGTA, and EDTA (Nacalai Tesque, Inc., Kyoto, Japan); creatine phosphate (Calbiochem-Novabiochem, Co., Darmstadt, Germany); [^{14}C]-taurocholate (Amersham Biosciences Co., Piscataway, NJ). All other chemicals were of analytical grade.

Expression of ABCB11 in Sf9 Insect Cells. The cDNA of human ABCB11 (wild type) cloned from a human liver cDNA library was inserted into the pFastBac1 vector between the restriction enzyme sites of *Not*I and *Hin*dIII (Figure 1A). To express ABCB11 in insect cells, recombinant baculoviruses were generated with the Bac-to-Bac baculovirus expression systems (Invitrogen, Co., Carlsbad, CA) according to the manufacturer's instructions. Insect *Spodoptera frugiperda* Sf9 cells (1×10^6 cells/mL) were infected with the recombinant baculoviruses and cultured in EX-CELL 420 insect serum-free medium (JRH Bioscience, Levexa, KS) at 27 °C with gentle shaking. The expression of ABCB11 in Sf9 cells increased during the incubation (Figure 1B). Three days after the infection, the cells were harvested by centrifugation, subsequently washed with phosphate-buffered saline (PBS) at 4 °C, collected by centrifugation, and stored at −80 °C until used.

Preparation of the Plasma Membrane Vesicles from Sf9 Cells. Plasma membrane vesicles were prepared from ABCB11-expressing Sf9 cells as described previously.[20] The frozen cell pellet was thawed quickly, diluted 40-fold with a hypotonic buffer (0.5 mM Tris/HEPES, pH 7.4, 0.1 mM EGTA, and 1 μM leupeptin), and then homogenized with a Potter-Elvehjem homogenizer. After centrifugation at 2000*g*, the supernatant was further centrifuged at 100000*g* for 30 min. The resulting pellet was suspended in 0.25 M sucrose containing 10 mM Tris/HEPES, pH 7.4, and 1 μM leupeptin. The crude membrane fraction was layered over 40% (w/v) sucrose solution and centrifuged at 100000*g* for 30 min. The turbid layer at the interface was collected, suspended in 0.25 M sucrose containing 10 mM Tris/HEPES, pH 7.4, and centrifuged at 100000*g* for 30 min. The membrane fraction was collected and resuspended in a small volume (150−250 μL) of 0.25 M sucrose containing 10 mM Tris/HEPES, pH 7.4. After the protein concentration was measured by using the BCA protein assay kit (PIERCE Biotechnology, Inc., Rockford, IL), the membrane solution was stored at −80 °C until used.

Immunological Detection of ABCB11 Expression in Plasma Membrane. Expression of ABCB11 in Sf9 cell membranes was determined by immunoblotting with C219 (Calbiochem-Novabiochem Co., Darmstadt, Germany), a

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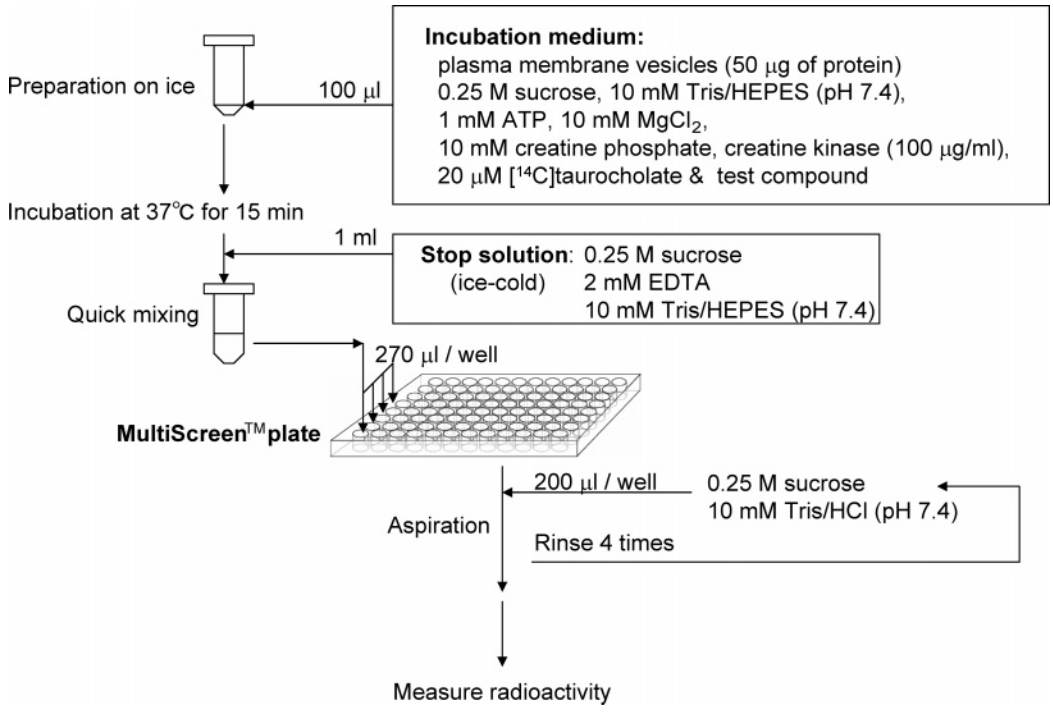

Figure 2. The procedure to measure ATP-dependent taurocholate transport mediated by ABCB11 in plasma membrane vesicles.

monoclonal antibody specific to human ABCB1 and ABCB11.[7] Briefly, proteins of the isolated plasma membrane were separated by electrophoresis on 7.5% sodium dodecyl sulfate (SDS) polyacrylamide slab gels, and the proteins were electroblotted onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences Co., Piscataway, NJ). Immunoblotting was performed by using C219 (1:100 dilution) as the first antibody and an anti-mouse IgG−horseradish peroxidase (HRP) conjugate (Cell Signaling Technology, Beverly, MA) (1:3000 dilution) as the second antibody. HRP-dependent luminescence was developed by using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences Inc., Boston, MA) and detected by a Lumino imaging analyzer FAS-1000 (TOYOBO Ltd., Osaka, Japan).

High-Speed Screening of ATP-Dependent [^{14}C]Taurocholate Transport Mediated by ABCB11. The frozen stocked membrane was thawed quickly at 37 °C, and vesicles were formed by passing the membrane suspension through a 27 gauge needle. Figure 2 depicts the scheme of the high-speed screening of ATP-dependent [^{14}C]taurocholate transport mediated by ABCB11. The standard incubation medium contained plasma membrane vesicles (50 μg of protein), 20 μM [^{14}C]taurocholate, 0.25 M sucrose, 10 mM Tris/HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM creatine phosphate, and 100 μg/mL creatine kinase in a final volume of 100 μL. The incubation was carried out at 37 °C. After a specified time (15 min for the standard condition), the reaction medium was mixed with 1 mL of ice-cold stop solution (0.25 M sucrose, 10 mM Tris/HEPES, pH 7.4, and 2 mM EDTA) to terminate the transport reaction. Subsequently, aliquots (270 μL per well) of the resulting mixture were transferred to MultiScreen plates (Millipore Co., Bedford, MA). Under aspiration, each well of the plate was rinsed four times with the 0.25 M sucrose solution containing 10 mM Tris/HCl, pH 7.4 (4 × 200 μL for each well), in an EDR384S system (BioTec Co., Tokyo, Japan). [^{14}C]Taurocholate thus incorporated into the vesicles was measured by counting the radioactivity remaining on the filter of MultiScreen plates, as described previously.[23]

QSAR Analysis Using Chemical Fragmentation Codes. To perform the QSAR analysis for the natural compounds and drugs tested in this study, we generated their chemical fragmentation codes by using the Markush TOPFRAG program (Derwent Information, Ltd., London, U.K.).[21] The chemical fragmentation codes are a set of alphanumeric symbols, each representing a fragment of a chemical structure. The Markush TOPFRAG program is a tool for searching the chemical structures and structure information in Derwent's online databases.[22] In the present study, the chemical fragmentation coefficient is defined as the contribution to the activity (here the ability to inhibit taurocholate transport) that is attributable to the presence of a particular chemical moiety in the test compound. We have formulated the extent of inhibition of ABCB11-mediated taurocholate transport as a linear combination of chemical fragmentation codes, each of which was weighted by the corresponding coefficient as follows:

$$\text{ABCB11 inhibition (\%)} = \sum C(\text{i}) \times \text{score(i)} + \text{constant}$$

where the symbol (i) designates a specific chemical frag-

(21) http://scientific.thomson.com/support/patents/patinf/terms/; accessed on April 25, 2006.

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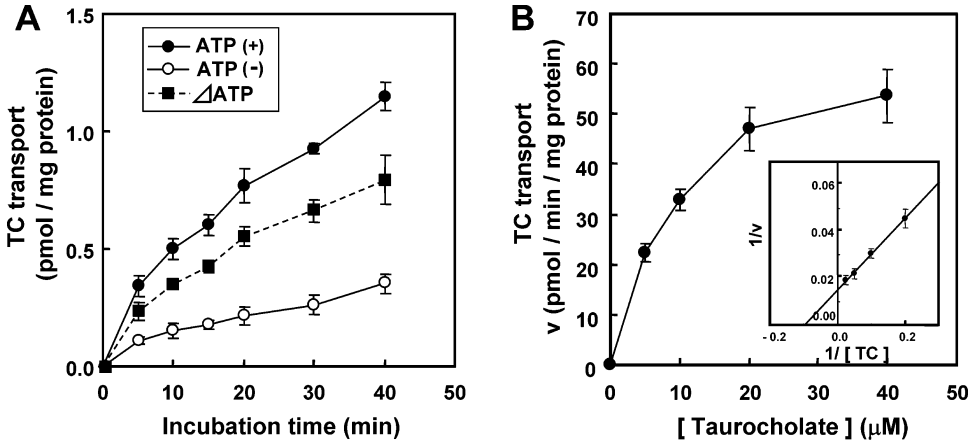

Figure 3. (A) Time courses of ATP-dependent transport of taurocholate in plasma membrane vesicles. ABCB11-expressing plasma membrane vesicles (50 μg of protein) were incubated with 20 μM [^{14}C]taurocholate in the presence (●) or absence (○) of 1 mM ATP in the standard incubation medium (0.25 M sucrose, 10 mM Tris/HEPES, pH 7.4, 10 mM creatine phosphate, 100 μg/mL creatine kinase, and 10 mM $MgCl_2$) at 37 °C. The amount of taurocholate transported into membrane vesicles was detected as described in the Experimental Section. ATP-dependent transport (■) was calculated from the difference between the amounts of [^{14}C]taurocholate transported into vesicles in the presence or absence of ATP. (B) The effect of taurocholate concentration on the rate of ABCB11-mediated taurocholate transport. ABCB11-expressing plasma membrane vesicles (50 μg of protein) were incubated with [^{14}C]taurocholate at different concentrations in the standard incubation medium at 37 °C for 15 min. The Michaelis−Menten constant was determined from the Lineweaver−Burk plot (inset). TC: taurocholate.

mentation (i). The "score" means the presence [≥1] or absence [0] of the corresponding chemical fragmentation code (i) in the chemical structure of a test compound. On the basis of the chemical fragmentation codes thus obtained and in comparison with the observed inhibition of transport activity for each test compound, we have calculated chemical fragmentation coefficients, C(i), by multiple linear regression analysis, as described previously.[23]

Results

Characterization of ATP-Dependent Transport of Taurocholate Mediated by Human ABCB11 in Plasma Membrane Vesicles. Sf9 cells (1×10^6 cells/mL) were infected with ABCB11-recombinant baculovirus and cultured at 27 °C with gentle shaking. Figure 1B shows immunoblot analysis data, where the expression of human ABCB11 in Sf9 cells increased during the incubation. The cell size and morphorogy of the infected cells changed dramatically, as detected by scanning electron microscopy (data not shown). The cell viability started to decrease after day 4. Since the integrity of cell membranes is critical for the following experiments of membrane vesicle transport, we harvested Sf9 cells on day 3 where cell viability was maintained at high levels (>90%).

We measured ATP-dependent transport of [^{14}C]taurocholate by using the plasma membrane vesicles prepared from ABCB11-expressing Sf9 cells. [^{14}C]Taurocholate was transported into the membrane vesicles in an ATP-dependent manner (Figure 3A), whereas such ATP-dependent transport of taurocholate was not observed in plasma membrane vesicles prepared from mock virus-infected Sf9 cells (data not shown). ABCB11-mediated taurocholate transport exhibited saturation kinetics (Figure 3B). From the Lineweaver−Burk plot (Figure 3B inset), the apparent K_m value was estimated to be 10.2 μM for taurocholate.

Inhibition of ABCB11-Mediated Taurocholate Transport. By using the high-speed screening system developed in our laboratory,[20] we have investigated the interaction of ABCB11 with a variety of test compounds. Figure 4A shows dose-dependent curves for the inhibition of ABCB11-mediated taurocholate transport by glibenclamide, rapamycin, rifampicin, and troglitazone. ATP-dependent [^{14}C]taurocholate transport in plasma membrane vesicles was measured at 37 °C for 15 min in the presence of those test compounds at different concentrations. IC_{50} values were determined to be 60, 11, 120, and 25 μM for glibenclamide, rapamycin, rifampicin, and troglitazone, respectively. The structures of those drugs are demonstrated in Figure 4B. Rapamycin and rifampicin have macrolide-like ring structures, whereas taurocholate, glibenclamide, and troglitazone do not.

We selected structurally diverse test compounds to examine our hypothesis that common chemical components may be involved in the inhibition of ABCB11-mediated taurocholate transport. The selected test compounds were classified into seven groups, i.e., A, neurotransmitters; B, calcium channel blockers; C, potassium channel modulators; D, steroids; E, nonsteroidal antiinflammatory drugs (NSAIDs); F, anticancer drugs; and G, miscellaneous. Figure 5 summarizes the effects of those test compounds on ABCB11-mediated taurocholate transport. The test compounds were measured at a concentration of 100 μM, and the data are

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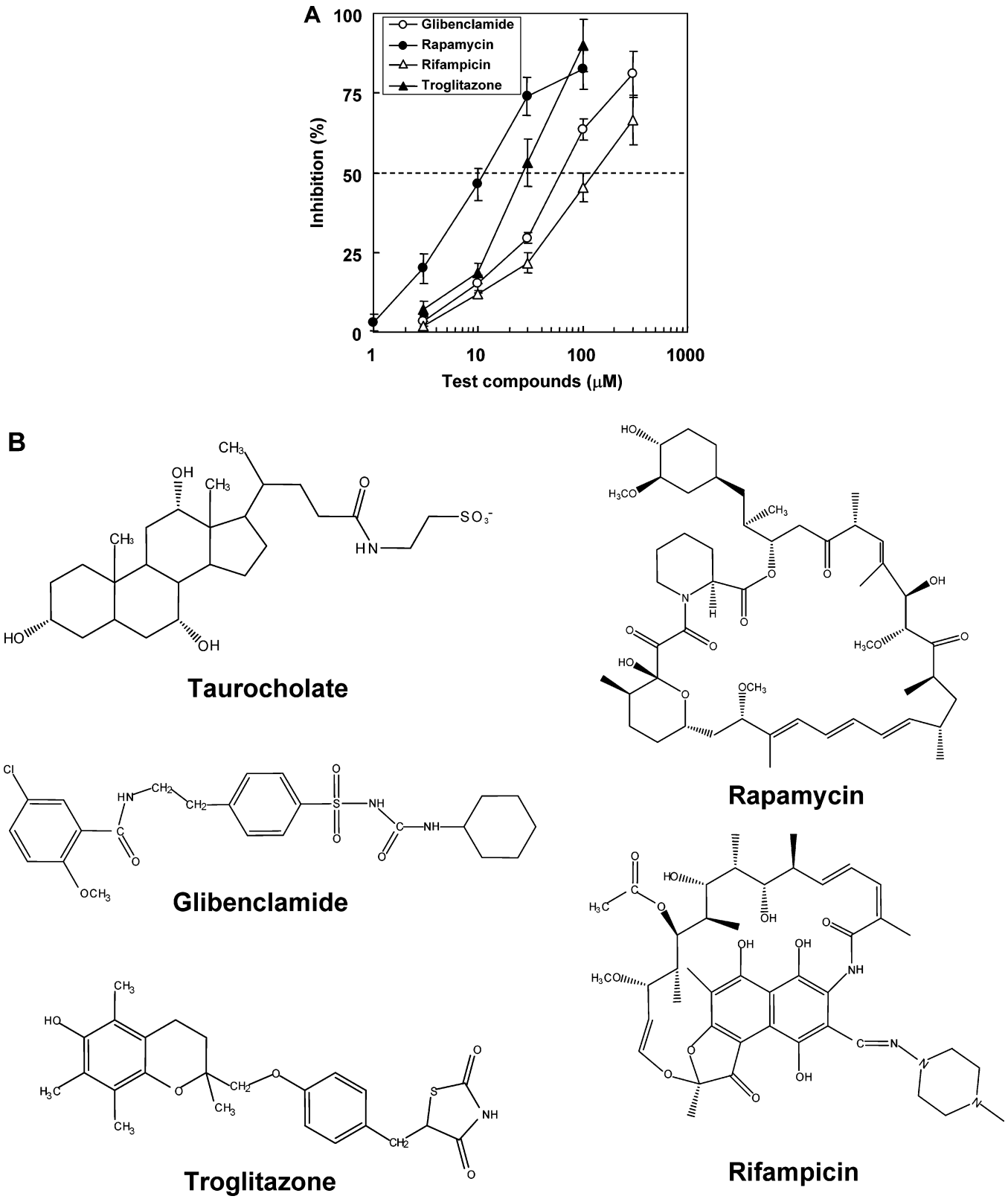


Figure 4. (A) Inhibition of ABCB11-mediated taurocholate transport by glibenclamide, rapamycin, rifampicin, and troglitazone. ABCB11-expressing plasma membrane vesicles (50 μg of protein) were incubated with 20 μM [^{14}C]taurocholate in the presence of a drug at different concentrations as indicated in the figure. (B) Chemical structures of glibenclamide, rapamycin, rifampicin, and troglitazone.

expressed as relative values as compared with the transport activity measured without test compounds (0% inhibition). Among 42 different therapeutic drugs and compounds tested in this study (Figure 6), nifedipine (B-2), fendiline (B-5),

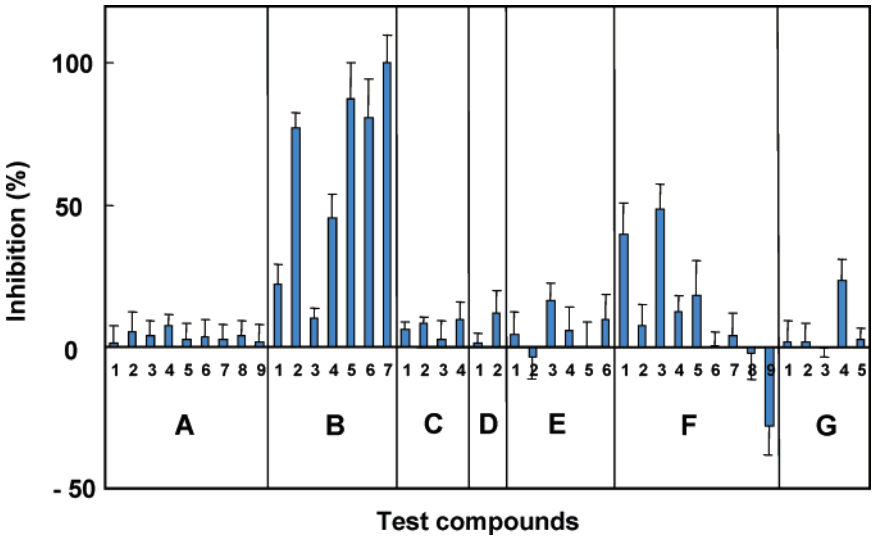


Figure 5. Inhibition of ABCB11-mediated taurocholate transport by test compounds and their profiling. ATP-dependent [^{14}C]-taurocholate transport was measured in the presence of a test compound (100 μM) in the standard incubation medium (0.25 M sucrose and 10 mM Tris/HEPES, pH 7.4, 10 mM creatine phosphate, 100 μg/mL creatine kinase, 10 mM $MgCl_2$), as described in the Experimental Section. Inhibition (%) is expressed as relative values compared with the transport activity measured without test compounds (0% inhibition). The test compounds used are classified into seven groups: (A) neurotransmitters, (B) calcium channel blockers, (C) steroids, (D) potassium channel modulators, (E) nonsteroidal antiinflammatory drugs, (F) anticancer drugs, and (G) miscellaneous. Test compounds: glycine (A-1), glutamic acid (A-2), dopamine (A-3), norepinephrine (A-4), epinephrine (A-5), GABA (A-6), histamine (A-7), serotonin (A-8), melatonin (A-9), verapamil (B-1), nifedipine (B-2), diltiazem (B-3), bepridil (B-4), fendiline (B-5), prenylamine (B-6), nicardipine (B-7), dexamethasone (C-1), betamethasone (C-2), prednisolone (C-3), cortisone (C-4), nicorandil (D-1), pinacidil (D-2), acetylsalicylic acid (E-1), indomethacin (E-2), acemetacin (E-3), ibuprofen (E-4), naproxen (E-5), mepirizole (E-6), vinblastine (F-1), etoposide (F-2), actinomycin D (F-3), daunorubicin (F-4), paclitaxel (F-5), methotrexate (F-6), doxorubicin (F-7), 5-fluorouracil (F-8), mitoxantrone (F-9), quinidine (G-1), *p*-aminohippuric acid (G-2), penicillin G (G-3), novobiocin (G-4), and prazosin (G-5).

prenylamine (B-6), and nicardipine (B-7) strongly inhibited ABCB11-mediated taurocholate transport (Figure 5). Bepridil (B-4), vinblastine (F-1), and actinomycin D (F-3) were moderate inhibitors. Interestingly, mitoxantrone (F-9) enhanced taurocholate transport, as indicated by the negative value of inhibition (%) in Figure 5.

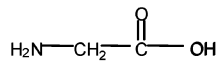
QSAR Analysis. To gain more insight into the relationship between the chemical structures of the test compounds and the inhibition of ABCB11-mediated taurocholate transport activity, we have performed a QSAR analysis by introducing chemical fragmentation codes. The program Markush TOPFRAG[21,22] was used to generate chemical fragmentation codes based on the structural components of the test compounds, as described previously.[23] In the present study, steroids (group C) and actinomycin D (F-3) were excluded from this analysis, because the Markush TOPFRAG program does not have an algorithm to generate chemical fragmentation codes for steroids and peptides. Multiple linear regression analysis delineated a relationship between the structural components and the extent of ABCB11 inhibition (Table 1). In this way, we could identify one set of chemical fragmentation codes that are closely related to the inhibition of ABCB11 transport activity. Explanations for these chemical fragmentation codes are also given in Table 1.

In this study, we used the descriptors of M132, ESTR, R-CC, H181, MN-HC, and OH-ALP to represent multiple chemical fragmentation codes. On the basis of the results of the multiple linear regression analysis, we calculated the values of predicted inhibition and compared them with the observed ones (Table 2). As demonstrated in Figure 7A, the prediction of transport inhibition correlated well with the observed values of inhibition. The R^2 value was estimated to be 0.952.

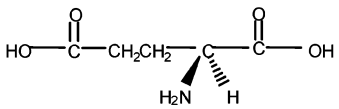
Discussion

High-Speed Screening and QSAR Analysis To Evaluate ABCB11−Drug Interactions. In the present study, we have developed a high-speed screening system and QSAR analysis method to analyze the inhibition of ABCB11-mediated taurocholate transport by test compounds. ABCB11 and ABCB1 (P-gycoprotein/MDR1) share about 50% identity in amino acid sequence and about 70% similarity. While ABCB11 is closely related to ABCB1, it is more selective in tissue distribution and substrate recognition. Because inhibition or loss of ABCB11 function has severe consequences, including intrahepatic cholestasis and hepatotoxicity, resulting from exposure to toxic xenobiotics or drug interactions, in vitro screening methods are necessary for quantifying and characterizing the inhibition of ABCB11. To discern the contribution of ABCB11 to drug interactions, it is critical to explore and demonstrate methods for characterizing and quantifying the inhibition of ABCB11-mediated transport. Byrne et al. have recently characterized the substrate specificity and inhibition of human ABCB11

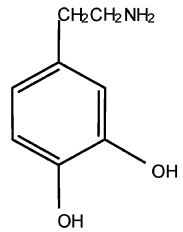
Group A Neurotransmitters



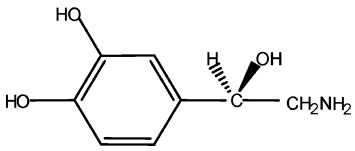
A-1 Glycine



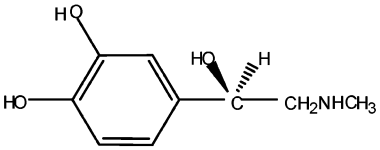
A-2 Glutamic acid



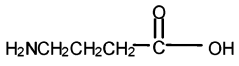
A-3 Dopamine



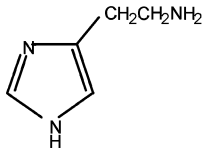
A-4 Norepinephrine



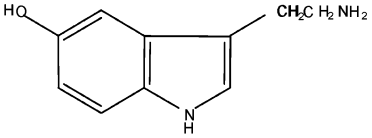
A-5 Epinephrine



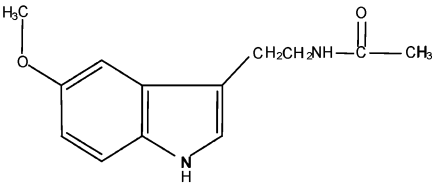
A-6 GABA



A-7 Histamine

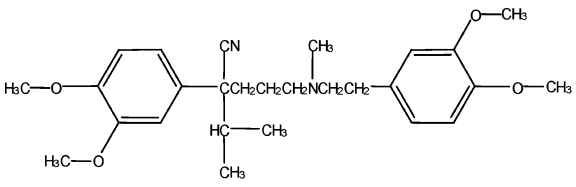


A-8 Serotonin

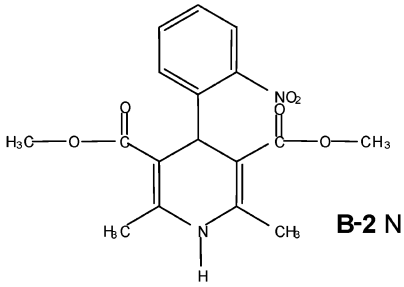


A-9 Melatonin

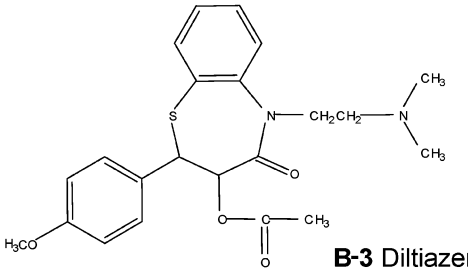
Group B Calcium channel blockers



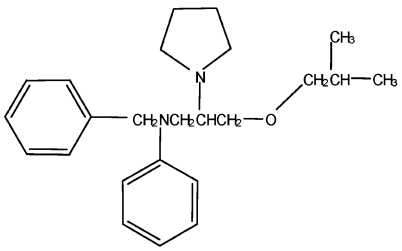
B-1 Verapamil



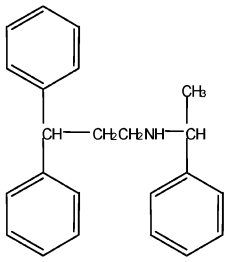
B-2 Nifedipine



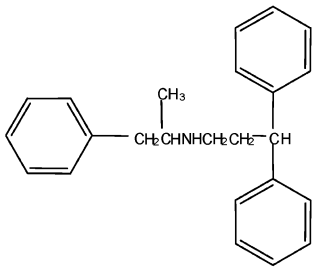
B-3 Diltiazem



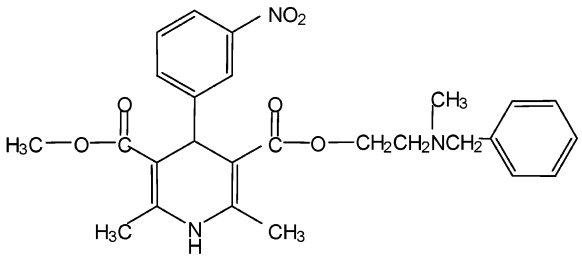
B-4 Bepridil



B-5 Fendiline



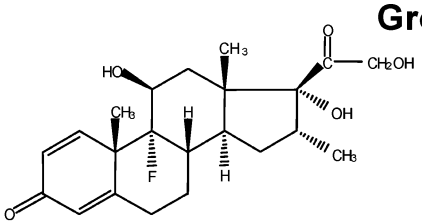
B-6 Prenylamine



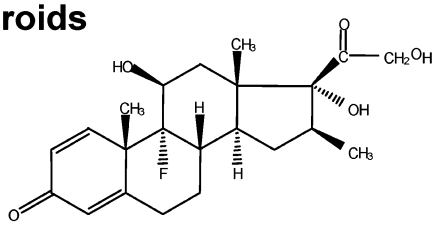
B-7 Nicardipine

Figure 6 (1 of 3)

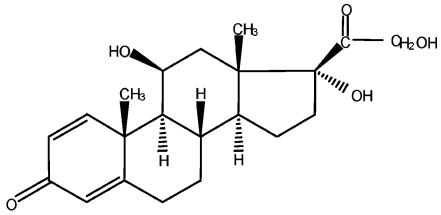
Group C Steroids



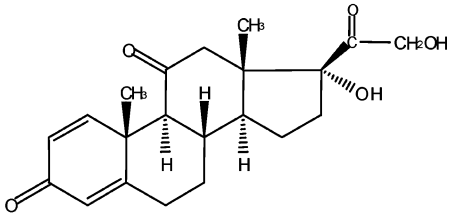
C-1 Dexamethasone



C-2 Betamethasone

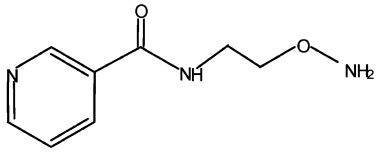


C-3 Prednisolone

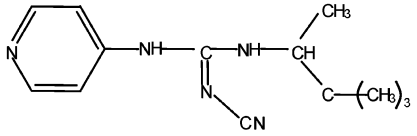


C-4 Cortisone

Group D Potassium channel modulators

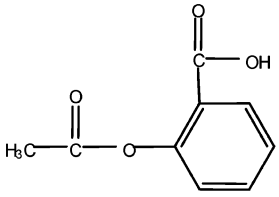


D-1 Nicorandil

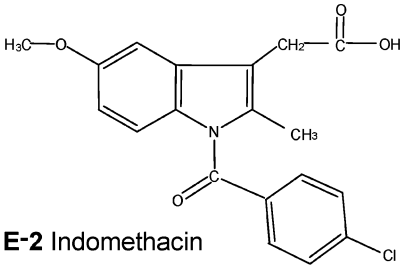


D-2 Pinacidil

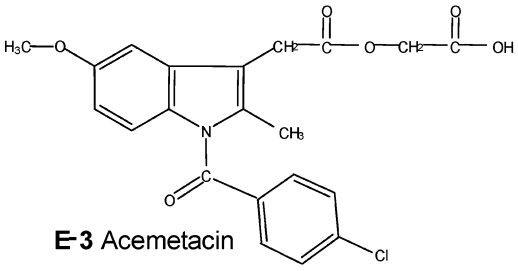
Group E Non-steroidal anti-inflammatory drugs



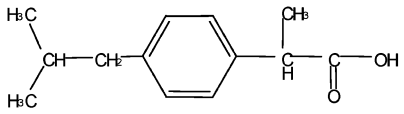
E-1 Acetylsalicylic acid



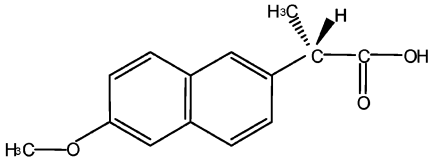
E-2 Indomethacin



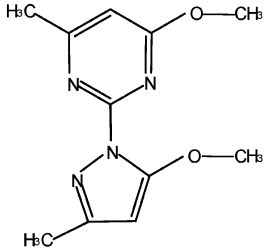
E-3 Acemetacin



E-4 Ibuprofen



E-5 Naproxen



E-6 Mepirizole

Figure 6 (2 of 3)

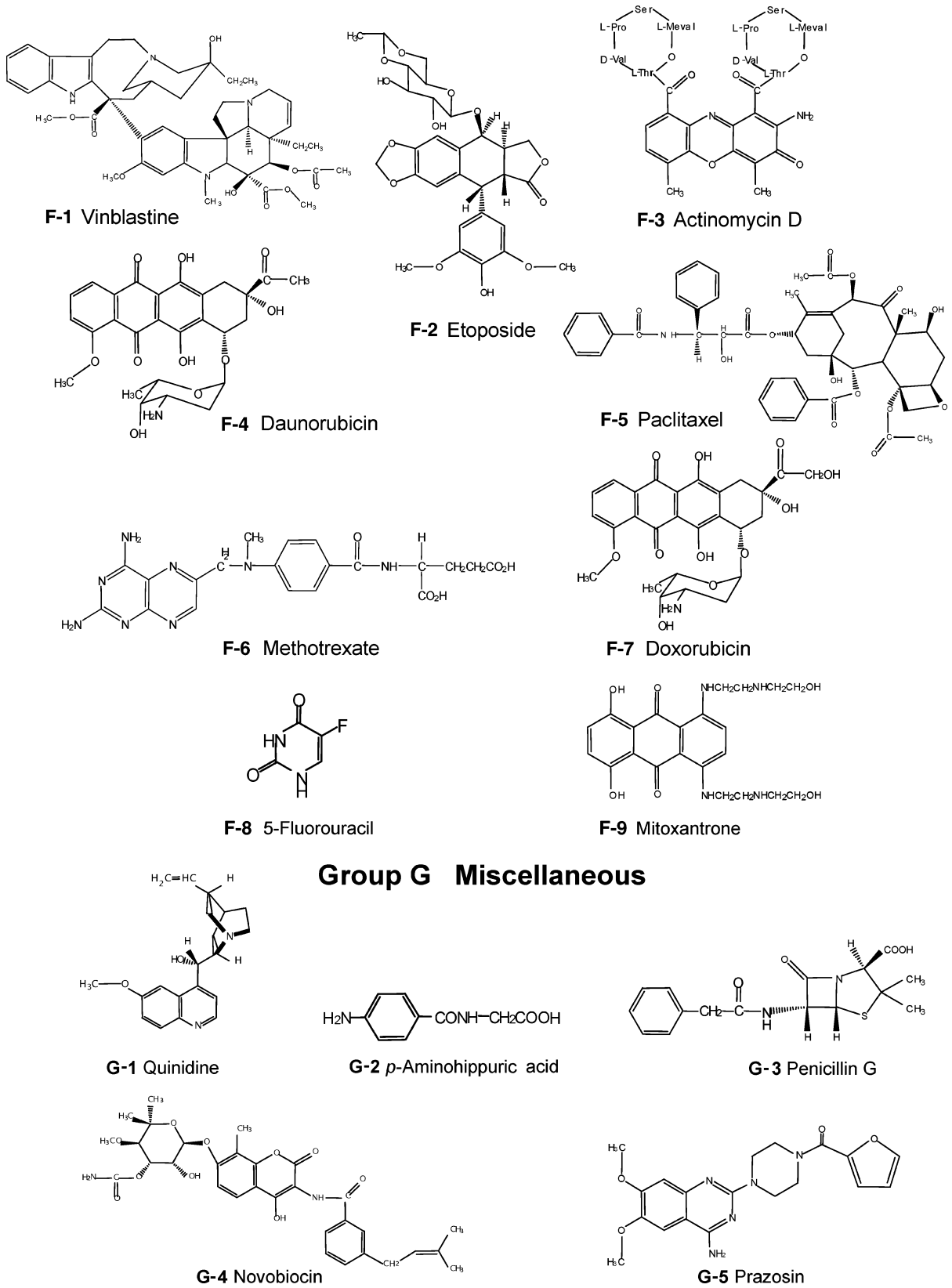


Figure 6. Chemical structures of test compounds used for the inhibition of ABCB11-mediated taurocholate transport.

by using plasma membrane vesicles prepared from ABCB11-expressing insect cells.[24] In their study, cyclosporin A, rifampicin, and glibenclamide were found to be competitive inhibitors. On the other hand, Wang et al. evaluated several

Table 1. Definition of Descriptors and Chemical Fragmentation Codes (CFC) Contributing to Inhibition of ABCB11-Mediated Taurocholate Transport[a]

descriptor	coefficient (95% reliability)		CFC	definition	score
M132	35.07	(±8.99)	M132	ring-linking group containing one C atom (except for M131 >C═W, W: heteroatom)	1
ESTR	31.47	(±4.97)	J211	one ester (thioester) group bonded to heterocyclic C via >C═O (>C═S)	1
			J212	two or more ester (thioester) groups bonded to heterocyclic C via >C═O (>C═S)	2
R-CC	14.41	(±2.91)	M530	no carbocyclic system with at least one aromatic ring	0
			M531	one carbocyclic system with at least one aromatic ring	1
			M532	Two carbocyclic systems with at least one aromatic ring	2
			M533	three or more carbocyclic systems with at least one aromatic ring	3
H181	10.95	(±4.86)	H181	one amine bonded to aliphatic C	1
MN-HC	9.66	(±4.22)	M520	no mononuclear heterocycle	0
			M521	one mononuclear heterocycle	1
			M522	two mononuclear heterocycles	2
			M523	three or more mononuclear heterocycles	3
OH-ALP	−15.23	(±5.06)	H481	one OH group bonded to aliphatic C	1
			H482	two OH groups bonded to aliphatic C	2
			H483	three OH groups bonded to aliphatic C	3
			H484	four or more OH groups bonded to aliphatic C	4
constant	−9.50				1

[a] $R = 0.976$; $R^2 = 0.952$; $F(30,6) = 99.1$; $s = 6.67$. Descriptors, CFC, coefficients, and constants were deduced from the inhibition of ABCB11-mediated taurocholate transport by test compounds (Figure 5).

fluorescent compounds for interaction with ABCB11 and showed that dihydrofluorescein (H_2FDA), at least, was useful for evaluating ABCB11 inhibition and assessing drug interactions.[25]

To detect the transport activity of ABCB11 and its inhibition, we used membrane vesicles prepared from Sf9 insect cells highly expressing human ABCB11. Recently, we developed a high-speed screening system by introducing 96-well MultiScreen plates and an automated multidispenser system,[20] which made the assay speed 50 times faster than the original method. It is important to note that the inhibition profile for ABCB11 (Figure 5) is very similar to the ATPase profile for ABCB1. Calcium channel blockers, e.g., fendiline (B-5), prenylamine (B-6), and nicardipine (B-7), strongly inhibited ABCB11-mediated taurocholate transport. Those drugs stimulated the ATPase activity of ABCB1 expressed in the plasma membrane fraction from Sf9 insect cells,[23] suggesting a close relationship between ABCB11 and ABCB1 in terms of drug interactions. Bile salts are synthesized in the liver by the enzymatic modification of cholesterol, and comprise a steroid nucleus and an aliphatic side chain. However, steroids, such as dexamethasone (C-1), betamethasone (C-2), prednisolone (C-3), and cortisone (C-4), did not significantly inhibit ABCB11-mediated taurocholate transport (Figure 5). Taken together, these findings suggest that ABCB11 preferably recognizes bile salts as substrates.

To quantitatively analyze the ABCB11-inhibition screening data (Figure 5), we carried out a QSAR analysis to evaluate structural components associated with ABCB11−drug interactions. In the 1960s, Hansch first developed another QSAR analysis method.[26,27] Initially, Hansch and Fujita studied the role of octanol/water partition coefficients (log *P*) in drug transport processes that were thought to contribute to drug absorption and distribution in the body.[26−28] Since their pioneering work, log *P* has been recognized as the most predominant descriptor in many QSAR studies. Nevertheless, other approaches can be used to describe chemical structures in a quantitative approach.

Recently, we have developed a new QSAR analysis method to study the substrate specificity of ABCB1 (P-glycoprotein/MDR1), where we used chemical fragmentation codes to describe the chemical structures of a variety of drugs and natural compounds.[23] Derwent Information, Ltd., developed this structure-indexing language, which is suitable for describing chemical structures. The chemical fragmentation codes were originally created to answer the need for accessing information in the increasing numbers of chemical patents. Markush TOPFRAG is the software that generates the chemical fragmentation codes from chemical structure information.[21,22]

(24) Byrne, J. A.; Strautnieks, S. S.; Mieli-Vergani, G.; Higgins, C. F.; Linton, K. J.; Thompson, R. J. The human bile salt export pump: characterization of substrate specificity and identification of inhibitors. *Gastroenterology* **2002**, *123*, 1649−1658.

(25) Wang, E. J.; Casciano, C. N.; Clement, R. P.; Johnson, W. W. Fluorescent substrates of sister-P-glycoprotein (BSEP) evaluated as markers of active transport and inhibition: evidence for contingent unequal binding sites. *Pharm. Res.* **2003**, *20*, 537−544.

(26) Hansch, C. The physicochemical approach to drug design and discovery (QSAR). *Drug Dev. Res.* **1981**, *1*, 267−309.

(27) Hansch, C. Quantitative structure−activity relationships and the unnamed science. *Acc. Chem. Res.* **1993**, *26*, 147−153.

(28) Fujita, T. Application of quantitative structure-activity relationship in drug design. *Acta Pharm. Jugosl*. **1987**, *37*, 43−51.

Table 2. QSAR-Based Prediction and Comparison with Observed Values in the Inhibition of ABCB11-Mediated Taurocholate Transport

		descriptor and score							inhibition (%)	
test compounds		M132	ESTR	R-CC	H181	MN-HC	OH-ALP	constant	predicted	observed
A-1	glycine	0	0	0	1	0	0	1	1.45	1.03
A-2	glutamic acid	0	0	0	1	0	0	1	1.45	4.98
A-3	dopamine	0	0	1	1	0	0	1	15.86	3.82
A-4	norepinephrine	0	0	1	1	0	1	1	0.63	7.58
A-5	epinephrine	0	0	1	1	0	1	1	0.63	2.69
A-6	GABA	0	0	0	1	0	0	1	1.45	3.54
A-7	histamine	0	0	0	1	1	0	1	11.10	2.61
A-8	serotonin	0	0	0	1	0	0	1	1.45	3.69
A-9	melatonin	0	0	0	0	0	0	1	−9.50	1.55
B-1	verapamil	0	0	2	1	0	0	1	30.27	21.94
B-2	nifedipine	0	2	1	0	1	0	1	77.51	76.98
B-3	diltiazem	0	0	1	1	0	0	1	15.86	10.17
B-4	bepridil	0	0	2	1	1	0	1	39.92	45.45
B-5	fendiline	1	0	3	1	0	0	1	79.74	87.46
B-6	prenylamine	1	0	3	1	0	0	1	79.74	80.70
B-7	nicardipine	0	2	2	1	1	0	1	102.86	99.99
D-1	nicorandil	0	0	0	0	1	0	1	0.16	1.44
D-2	pinacidil	0	0	0	0	1	0	1	0.16	11.80
E-1	acetylsalicylic acid	0	0	1	0	0	0	1	4.91	4.26
E-2	indomethacin	0	0	1	0	0	0	1	4.91	−3.80
E-3	acemetacin	0	0	1	0	0	0	1	4.91	16.27
E-4	ibuprofen	0	0	1	0	0	0	1	4.91	5.60
E-5	naproxen	0	0	1	0	0	0	1	4.91	0.08
E-6	mepirizole	0	0	0	0	2	0	1	9.82	9.40
F-1	vinblastine	0	1	0	1	0	0	1	32.92	39.67
F-2	etoposide	0	0	1	0	0	0	1	4.91	7.40
F-4	daunorubicin	0	0	1	0	1	0	1	14.57	12.36
F-5	paclitaxel	0	0	3	0	0	1	1	18.51	18.09
F-6	methotrexate	0	0	1	0	0	0	1	4.91	0.27
F-7	doxorubicin	0	0	1	0	1	1	1	−0.66	3.99
F-8	5-fluorouracil	0	0	0	0	1	0	1	0.16	−2.48
F-9	mitoxantrone	0	0	1	0	0	2	1	−25.54	−27.83
G-1	quinidine	1	0	0	0	0	1	1	10.34	1.67
G-2	*p*-aminohippuric acid	0	0	1	0	0	0	1	4.91	1.84
G-3	penicillin G	0	0	1	0	0	0	1	4.91	−0.75
G-4	novobiocin	0	0	1	0	1	0	1	14.57	23.22
G-5	prazosin	0	0	0	0	2	0	1	9.82	2.72

We have applied these chemical fragmentation codes to the QSAR analysis of ABCB11−drug interactions. Our approach is unique in that the extent of ABCB11-mediated taurocholate transport inhibition is described as a linear combination of chemical fragmentation codes, and that the coefficient "*C*(i)" for each chemical fragmentation code reflects the extent of the contribution of a specific chemical moiety to interactions with ABCB11 protein (see Experimental Section). It is also possible to further expand this QSAR analysis with a larger number of structurally diverse compounds, including drug metabolites as well.

The present study demonstrates that the structural components represented by the chemical fragmentation codes of M132 and H181 as well as by the descriptors of ESTR, R-CC, and MN-HC positively contributed to the inhibition, whereas the descriptor of OH-ALP (OH groups bonded to aliphatic carbon) had a negative contribution. As summarized in Table 1, the descriptor ESTR including chemical fragmentation codes of J211 and J212 had a relatively large positive coefficient, $C(\text{ESTR}) = 31.47$, suggesting that an ester (thioester) group bonded to a carbon of heterocyclic ring(s) is an important component for the interaction with the ABCB11 protein. In addition, the data for R-CC (Table 1) suggest that carbocyclic systems with at least one aromatic ring are also important chemical moieties for the interaction with ABCB11. These QSAR profiles are distinct from those for ABCG2 (Saito, H., et al., unpublished data). In the case of troglitazone, the chemical fragmentation codes of M132,

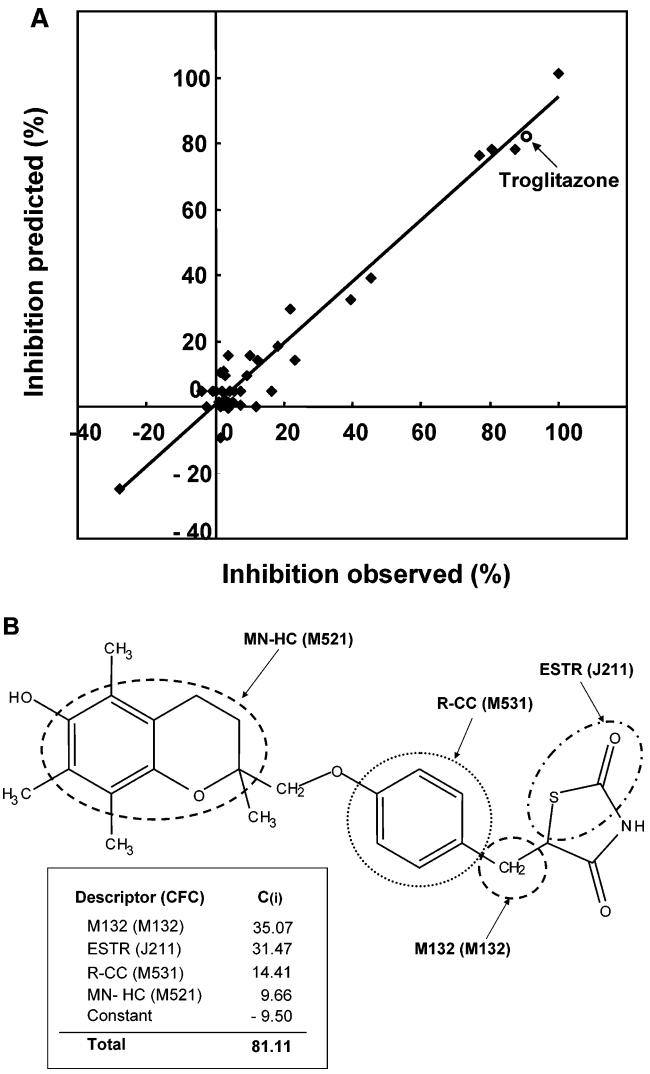


Figure 7. (A) Relationship between observed and predicted values in the inhibition of taurocholate transport by the test compounds described above. Those observed values were the same as shown in Figure 5, whereas the predicted values were deduced from the multiple linear regression analysis as described in the Experimental Section. The inhibition by troglitazone is indicated by an open circle (○). (B) Chemical structure of troglitazone and its descriptors and chemical fragmentation codes (CFC) associated with the inhibition of ABCB11-mediated taurochalate transport.

J211, M531, and M521 are involved in its chemical structure. Structural components corresponding to those chemical fragmentation codes are indicated in Figure 7B. On the basis of the QSAR calculation [ABCB11 inhibition (%) = *C*(M132) + *C*(J211) + *C*(M531) + *C*(M521) + constant], the inhibition of taurocholate transport by troglitazone is estimated to be 81.11%, which is in accordance with the observed value of 90.06% (Figures 4 and 7A).

Application of QSAR to Drug Discovery and Development. Inhibition of ABCB11 is a potential mechanism for the development of several acquired cholestatic liver diseases, including drug-induced intrahepatic cholestasis. Taurocholate transport by rat Abcb11 was competitively inhibited by the immunosuppressive drug cyclosporin A, the sulfonylurea antidiabetic drug glibenclamide,[29] and the endothelin receptor antagonist bosentan.[30] Use of the insulin sensitizer troglitazone for the treatment of type-2 diabetes has been shown to inhibit taurocholate transport in vivo as well as in canalicular membrane vesicles prepared from the rat liver.[17] It has been reported that troglitazone sulfate was efficiently formed and accumulated in liver tissue and that it strongly inhibited rat Abcb11 (IC_{50} value of 0.4−0.6 μM). Therefore, it is suggested that troglitazone sulfate is responsible for the interaction with hepatobiliary export of bile salts in rats. On the basis of those animal model and in vitro studies, inhibition of ABCB11 by troglitazone sulfate has been implicated as a potential cause for troglitazone-induced intrahepatic cholestasis in humans.

Our test compound library used for the ABCB11 inhibition in the present study did not include drug metabolites, such as troglitazone sulfate. In this context, we were not able here to analyze the inhibition of human ABCB11 by troglitazone sulfate. To improve the accuracy of our QSAR analysis, we would need to expand the test compound library and the data sets by incorporating structurally diverse drug candidates and lead compounds as well as their metabolites. In particular, drug candidates that were dropped out of development because of hepatotoxicity in the preclinical stage of drug discovery would be of utmost importance for our QSAR analysis to predict the drug-induced intrahepatic cholestasis.

Bile acids are of importance not only for the generation of bile flow but also for the regulation of a variety of genes via their interaction with the fernesoid X receptor (FXR)/bile acid receptor. Accumulating evidence suggests that disruption of bile salt homeostasis leads to unexpected interactions among nuclear hormone receptors, drug-metabolizing enzymes, and drug transporters.[5,31−35] In this context, the prediction and circumvention of ABCB11 inhibition by a drug or its metabolite(s) is a great challenge in drug discovery and development.

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Importance of ADME/Tox Screening in Basic Sciences. The NIH in the United States has recently implemented the Molecular Libraries Initiative (MLI) as a major component of the road map for medical research.[36] The goal of the MLI is to develop small-molecule modulators of cellular targets. In addition, the MLI will develop technology in four critical areas: namely, chemical diversity, assay diversity, instrumentation, and predictive ADME (absorption, distribution, metabolism, and excretion)/toxicology. There is a strong need for new approaches to determine the function and therapeutic potential for genes in the newly sequenced human genome as well as to accelerate the translation of basic research discoveries into new therapeutics. It is critically important to expand the availability, the flexibility, and the use of small-molecule chemical probes for basic research. Data sets and analysis algorithms for improved prediction of ADME and toxicity properties of small molecules will be developed in the ADME/toxicology area of the MLI.

In line with such initiatives, we developed the high-speed screening system to evaluate ABCB11−drug interactions in the present study. On the basis of the inhibition data, we could analyze the QSAR to identify one set of chemical fragmentation codes closely linked with the inhibition of ABCB11. By expansion of our test compound library and data sets including drug metabolites, we would be able to more accurately evaluate their interaction with ABCB11-mediated taurocholate transport. In the near future, this approach could be used to predict compounds with a potential risk of causing intrahepatic cholestasis.

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