High-Speed Screening and Quantitative SAR Analysis of Human ABC Transporter ABCG2 for Molecular Modeling of Anticancer Drugs to Circumvent Multidrug Resistance

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Abstract: The transport mechanism-based molecular design strategy would provide an effective tool for rationalized chemotherapy against tumors. To develop a platform for molecular modeling to circumvent multidrug resistance, we established new methods of high-speed screening for human ABCG2-drug interactions, quantitative structure-activity relationship (QSAR) analysis, and quantum chemical calculation for lead optimization.

Key Words: ABC transporter, ABCG2, BCRP, camptothecin, high-throughput screening, irinotecan, multidrug resistance, QSAR analysis.

1. INTRODUCTION

In the 21st century, emerging genomic technologies are shifting the paradigm of drug discovery and development. During this shifting phase, however, drug discovery and development is still remaining as high-risk, high-stakes ventures with long and costly timelines. Indeed, the attrition of drug candidates in preclinical and clinical stages is a major problem in drug discovery and development. In at least thirty percent of the cases, this attrition is due to poor pharmacokinetics (e.g., limited absorption. low plasma concentration levels, high rates of clearance). In the past, pharmaceutical companies have considered such early stage attrition an inevitable cost of doing business; however, as drug development costs have rocketed upward, pharmaceutical companies have begun to seriously re-evaluate their current strategies of drug discovery and development [1]. Thus, development of new innovative strategies is critically required to improve the success rate of drug discovery.

Evidence is accumulating to strongly suggest that drug transporters are one of the determinant factors governing the pharmacokinetic profile of drugs. Hitherto a variety of drug transporter genes have been cloned and classified into either solute carriers (SLC) or ATP-binding cassette (ABC) transporters. Such drug transporters are expressed in various tissues such as the intestine, brain, liver, kidney, and importantly cancer cells to play critical roles in the absorption, distribution and excretion of drugs. In that light, we have proposed that a 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 [2].

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2. AIM OF THIS REVIEW

Our strategy sprang from the realization that the processes of drug discovery and development are dramatically changing in conjunction with the introduction of new research technologies such as bioinformatics, functional genomics, and pharmacogenomics and their use to identify both classical drug targets (e.g., enzymes, membrane-bound receptors, and ion channels) and novel drug targets (e.g., cellular components of signal transduction, nuclear receptors, mRNA, and DNA). As a result, transport mechanism-based drug molecular design would become important in ensuring the site selectivity and pharmacological activity while reducing the side effects of new molecular drug candidates aimed at those targets. Recently, the importance of ATP-dependent drug transporters expressed in various tissues and cell types including cancer cells has been well recognized. In this regard, we expect that molecular design based on the drug transport mechanisms would improve the effectiveness of anticancer drugs and can contribute to the development of resistance-reversal agents or new anticancer drugs circumventing multidrug resistance of cancer.

To create such a platform of transport mechanisms-based molecular modeling, we have taken the new initiatives of high-speed screening to analyze transporter-drug interactions, as well as quantum chemical calculation and neural network quantitative structure-activity relationship (QSAR) analysis. This review presents our recent activities on the high-speed screening and QSAR analysis for human ABC transporter ABCG2 as well as a new strategy of molecular design for anticancer drugs to circumvent ABCG2-associated drug resistance.

3. BACKGROUNDS AND RATIONALE

Cancer is one of the gene-associated diseases, involving multiple factors in its cause and development. Despite enormous efforts spent on the development of cancer chemotherapies, these therapies are often effective only in a relatively small proportion of cancer patients. Acquired and intrinsic

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drug resistance in cancer is the major obstacle to long-term, sustained patient response to chemotherapy. It has been long recognized that the effectiveness of anticancer drugs can vary significantly among individual patients. Cancer cells appear to have the capacity to generate variants resistant to anticancer drugs, as part of their biological responses to external challenges. Tumors, and even individual cancer cells, can exhibit multiple mechanisms of resistance simultaneously.

There is accumulating evidence that active export of anticancer drugs from cells is one of the major mechanisms of drug resistance. It has been convincingly documented that several ATP-dependent drug transporters can cause drug resistance in cancer cells by actively extruding the clinically administered chemotherapeutic drugs. By far the well-known major drug transporters, i.e., ABCB1 (P-glycoprotein or MDR1), ABCC1 (MRP1), ABCC2 (MRP2, cMOAT), and ABCG2 (BCRP/ MXR/ABCP), have been characterized in detail with respect to their structure and function [3-6]. These drug transporters belong to the human ABC transporter gene family that consists of 48 gene members [3, 7-10].

4. ABCG2 (BCRP/MXR/ABCP)

ABCG2 is classified in the G-subfamily of the human ABC transporter gene family. This ABC transporter was originally named Breast Cancer Resistant Protein (BCRP), has recently been discovered in doxorubicin-resistant breast cancer cells [11]. Since the same transporter has also been found in the human placenta [12] as well as in drug-resistant cancer cells selected in mitoxantrone [13], the transporter was also called ABCP or MXR1. Compared with the molecular structures of ABCB1 and ABCC1, this ABC transporter is a so-called "half ABC transporter" bearing six transmembrane domains and one ATP-binding cassette. Recently, we have found that the ABCG2 protein forms homodimers *via* a cysteinyl disulfide bond at Cys603 under physiological conditions [14]. In fact, treatment with mercaptoethanol reduced the apparent molecular weight of ABCG2 from 140,000 to 70,000 [14, 15].

The *ABCG2* gene is located on chromosome 4q22 and spans over 66 kb, comprising of 16 exons and 15 introns [16]. The *ABCG2* gene is amplified or involved in chromosomal translocations in cancer cell lines selected with mitoxantrone, topotecan, or doxorubicin treatment, and ABCG2 was shown to confer resistance to anticancer drugs [17-22]. Several reports suggested that overexpression of ABCG2 is related to cancer cell resistance to camptothecin-based anticancer drugs, such as topotecan [23] and 7-ethyl-10-hydroxycamptothecin (SN-38: active metabolite of irinotecan, CPT-11) [24-26]. With this respect, we demonstrated that plasma membrane vesicles prepared from SN-38-resistant PC-6/SN2-5H2 human lung carcinoma cells ATP-dependently transported both SN-38 and SN-38-glucuronide [25, 26] to provide evidence that ABCG2 is involved in the active extrusion of SN-38 and its metabolite from cancer cells.

5. HIGH-SPEED SCREENING OF ABCG2-DRUG INTERACTIONS

To assess the substrate specificity of ABCG2 and its interactions with a variety of drugs including anticancer drugs, we measured ABCG2-mediated ATP-dependent transport by means of the vesicle transport assay method [27]. The original method for the vesicle transport assay was developed by Ishikawa to measure ATP-dependent transport of glutathione S-conjugates [28]. We recently improved the method to enhance the assay speed by introducing 96-well MultiScreen^{TM} plates and an automated multi-dispenser system which made the assay speed fifty-times faster than the original method [29]. To detect the transport activity of ABCG2, we used methotrexate (MTX) as a model substrate in our screening. The wild type of ABCG2 transports MTX, whereas acquired mutants, i.e., R482G and R482T, do not [15]. As compared with other substrates such as estrone-3-sulfate (E_3S), MTX was preferable for the high-speed screening because of the high signal-to-noise (S/N) ratio in our vesicle transport assay [29]. In addition, MTX can be easily replaced by inhibitors/substrates at the binding site(s) of ABCG2, because of its low affinity toward ABCG2. This is a great advantage for performing the inhibition screening and QSAR analysis.

By using the high-speed screening system, we have investigated the interaction of ABCG2 with a variety of test compounds (see Fig. (**1**) for the schematic diagram). For this purpose, we selected structurally diverse test compounds to investigate the inhibition of ABCG2-mediated MTX transport. The selected test compounds are classified into seven groups, i.e., A, neurotransmitters; B, Ca^{2+} channel blockers; C, K^+ channel modulators; D, steroids; E, non-steroidal anti-inflammatory drugs (NSAIDs); F, anti-cancer drugs; G, antibiotics; H, other drugs; and I, ABCG2 substrates or inhibitors. Fig. (**2A**) summarizes the effects of those test compounds on ABCG2-mediated MTX transport. The test compounds were measured at a concentration of 10 μM, and the data are expressed as relative values as compared with the transport activity measured without test compounds (0% inhibition). Among 49 different therapeutic drugs and compounds tested in this study, SN-38 (F-8), novobiocin (G-2), prazosin (H-4), tacrolimus (H-5), hematoporphyrin (I-3), pheophorbide a (I-4), and quercetin (I-5) strongly inhibited ABCG2-mediated MTX transport.

6. QSAR ANALYSIS USING CHEMICAL FRAGMENTATION CODES

To gain more insight into the relationship between the chemical structure of test compounds and the inhibition of ABCG2-mediated MTX transport activity, we have performed a QSAR analysis by introducing chemical fragmentation codes. While the chemical fragmentation codes were originally created to answer the need for accessing the increasing numbers of chemical patents, we apply them to our QSAR analysis. Derwent Information Ltd. developed the chemical fragmentation codes as a structure-indexing language that is suitable for describing chemical structures. The program Markush TOPFRAG is used to generate the chemical fragmentation codes based on the structural components of test compounds, as described previously [27, 29].

We applied this new approach to the QSAR analysis of ABCG2-drug interactions [29]. The uniqueness of our approach resides in the fact that the extent of ABCG2-mediated MTX transport inhibition is described as a linear combination of chemical fragmentation codes, and that the coeffi-

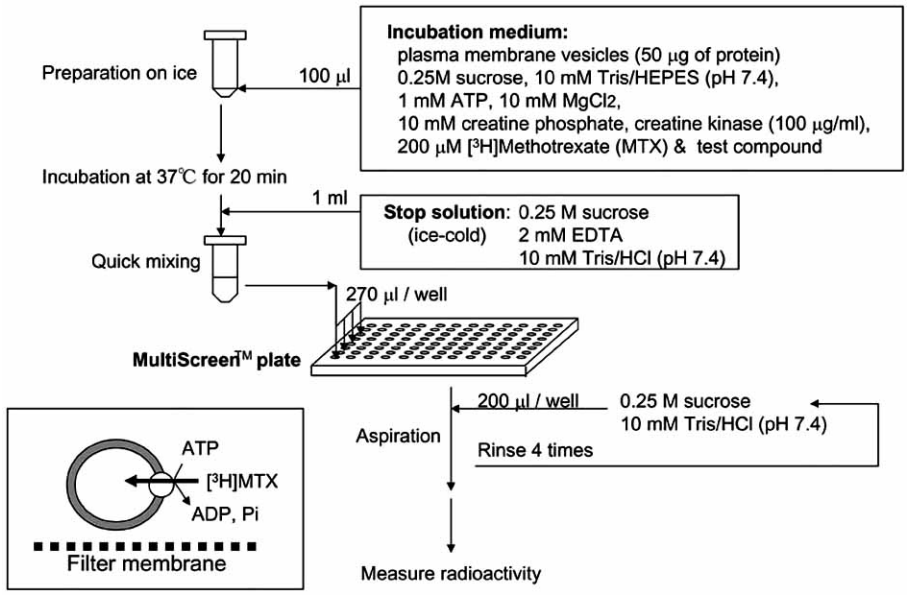
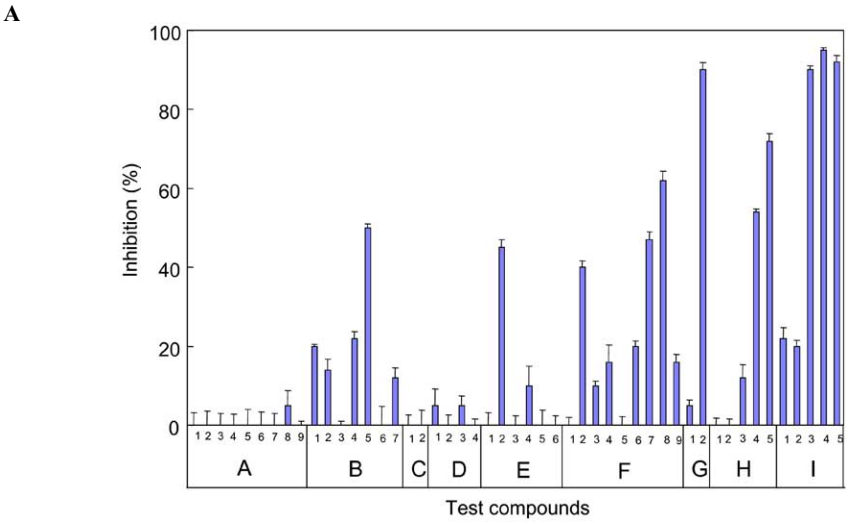


Fig. (1). Schematic illustration of high-speed screening of human ABCG2-drug interactions. Plasma membrane vesicles were prepared from ABCG2-expressing Sf9 cells. The standard incubation medium contained plasma membrane vesicles (50 μg of protein), 200 μM ^{3}H-labeled MTX, 0.25 M sucrose, 10 mM Tris/Hepes, 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 (20 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 (280 μl per well) of the resulting mixture were transferred to MultiScreen™ plates. Under aspiration, each well of the plate was rinsed with the 0.25 M sucrose solution containing 10 mM Tris/Hepes, pH 7.4, for four times (4 x 200 μl for each well) in an EDR384S system (BioTec, Tokyo, Japan). [^{3}H]MTX thus incorporated into the vesicles was measured by counting the radioactivity remaining on the filter of MultiScreen™ plates [Saito *et al*. 2006].

cient for each chemical fragment code reflects the extent of the contribution of a specific chemical moiety to interactions with ABCG2 protein. Multiple linear regression analysis delineated a relationship between the structural components and the extent of ABCG2 inhibition. Thereby we could identify one set of chemical fragmentation codes that are closely related to the inhibition of ABCG2 transport activity (Table **1**). Explanations for these chemical fragmentation codes are given in Table **2**. We use the descriptor of "OH" to represent five different chemical fragmentation codes for different numbers of hydroxyl (-OH) groups. Likewise, the descriptor of "RS4" is used to represent eighteen chemical fragmentation codes for various ring systems. Based on the results of the multiple linear regression analysis, we calculated the values of predicted inhibition and compared them with the observed ones (Table **3**). As demonstrated in Fig. (**2B**), the prediction of transport inhibition was well correlated with the observed values of inhibition. The R^2 value was estimated to 0.920.

Our QSAR analysis revealed that the structural components represented by the chemical fragmentation codes of



(Fig. 2. Contd….)

B

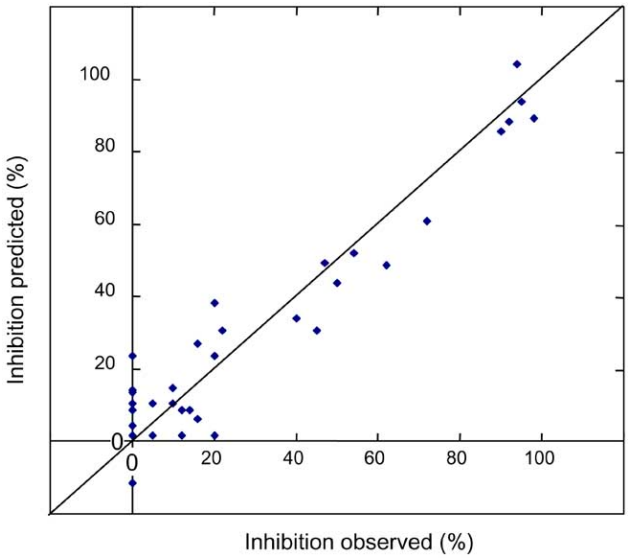


Fig. (2). Inhibition of MTX transport by test compounds and its profiling (A) as well as The relationships between observed and predicted values in the inhibition of MTX transport by different test compounds (B). A, ATP-dependent [^{3}H]MTX transport was measured in the presence of a test compound (10 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 Materials and Methods. Inhibition (%) is expressed as relative values compared with the transport activity measured without test compounds (0% inhibition). The test compounds used are: dopamine (A-1), epinephrine (A-2), norepinephrine (A-3), GABA (A-4), glutamic acid (A-5), glycine (A-6), histamine (A-7), melatonin (A-8), serotonin (A-9), bepridil (B-1), diltiazem (B-2), fendiline (B-3), nifedipine (B-4), nicardipine (B-5), prenylamine (B-6), verapamil (B-7), nicorandil (C-1), pinacidil (C-2), betamethasone (D-1), cortisone (D-2), dexamethasone (D-3), prednisolone (D-4), acetylsalicylic acid (E-1), acemetacin (E-2), indomethacin (E-3), ibuprofen (E-4), mepirizole (E-5), naproxen (E-6), actinomycin D (F-1), daunorubicin (F-2), doxorubicin (F-3), etoposide (F-4), 5-fluorouracil (F-5), mitoxantrone (F-6), paclitaxel (F-7), SN-38 (F-8), vinblastine (F-9), penicillin G (G-1), novobiocin (G-2), quinidine (H-1), *p*-aminohippuric acid (H-2), reserpine (H-3), prazosin (H-4), tacrolimus (H-5), estrone-3-sulfate (I-1), hemin (I-2), hematoporphyrin (I-3), pheophorbide a (I-4), and quercetin (I-5). Data are expressed as mean values ± S.E.M. (n = 3).

B, the observed inhibition values are correlated with the predicted values that were deduced from the multiple linear regression analysis.

H121, D023, and M240 as well as by the descriptor of OH positively contributed to the inhibition, whereas those of M531, J2, H481, and RS4 had negative contributions. As summarized in Table **1**, H121 had the largest positive coefficient, suggesting that one amine bonded to one carbon of a heterocyclic ring (Table **2**) is an important component for the interaction with the ABCG2 protein. In addition, the data for D023 (Table **2**) suggest that fused heterocyclic ring(s) and two substituents on a carbocyclic ring of the fused heterocyclic ring(s) are also important chemical moieties for the interaction with ABCG2.

Our QSAR analysis data (descriptor OH in Table **1**) suggest that polyphenols are potent inhibitors of ABCG2. Indeed, recent studies have shown that natural flavonoids, such as quercetin, genistein, naringenin, acacetin, and kaempferol, inhibit ABCG2 function and sensitize drug-resistant cancer cells *in vitro* [26, 30-34]. Co-administration of flavonoids with ABCG2-substrate anticancer drugs can alter the pharmacokinetic profile and consequently increase the efficacy of drugs. It is important to note that SN-38, an active metabolite of CPT-11, was a good substrate for ABCG2 and strongly inhibits ABCG2-mediated MTX transport. This is in accordance with our QSAR analysis data.

7. CPT-BASED ANTICANCER DRUGS

Camptothecin (CPT) was originally isolated from the wood of *Camptotheca acuminata*, a tree native to the rocky slopes of north China, isolated by Wall *et al.* [35]. Initial evidence of its antitumor activity was obtained in the screening of a large number of natural products by Drug Research and Development (formerly Cancer Chemotherapy National Service Center), National Cancer Institute (NCI). It was later found to have significant activity in mouse leukemia and rat Walker 256 carcinosarcoma. The possibility of usefulness against gastrointestinal carcinoma was originally raised because of the anti-proliferative effect of camptothecin in the intestinal mucosa of monkeys and beagles. This interest was intensified in 1970 by the phase I study of a group at the NCI Baltimore Cancer Research Center [36]. In that investigation, eight of nine patients with large bowel cancer were reported to have objective response.

In later studies, CPT has been demonstrated to be effective against a broad spectrum of tumors. The molecular target of CPT has been firmly established as being human DNA topoisomerase I (Topo I), which changes the topological state of duplex DNA by single-strand breakage and religation. Stabilization of the covalent Topo I-DNA complex (so-

Table 1. Descriptors, Coefficients, and Constant Deduced from the Inhibition of ABCG2-mediated MTX Transport by Test Compounds

Descriptor (*i*)	coefficient (*i*)	95% Reliability
H121	65.38	± 12.25
D023	50.10	± 8.99
M240	21.97	± 6.83
OH	12.40	± 3.10
M531	-13.06	± 6.66
J2	-19.99	± 8.33
H481	-31.64	± 11.72
RS4	-91.65	± 14.68
Constant	21.73	

Statistical significance was determined by *F*-test.

$R = 0.959$; $R^2 = 0.920$; $F = 50.1$; $n = 44$.

The F-value certifies that the QSAR equation is significant.

called "cleavable complex") by CPT is a critical step in its anti-tumor action where by Topo I-mediated DNA breaks are induced *via* prevention of DNA religation. CPT inhibits Topo I by blocking the rejoining step of the cleavage/reli-gation reaction of Topo I, resulting in the accumulation of a covalent Topo I-CPT-DNA intermediate, the cleavable complex [37-39]. Biochemical studies *in vitro* have revealed that CPT binds at the interface between Topo I and DNA and specifically inhibits the religation step in the cleavage/religation reaction [38, 40]. The molecular mechanism of inhibition appears to be of the uncompetitive type, because CPT binds neither the enzyme nor the DNA substrate, but interacts with the enzyme-DNA complex to form a reversible nonproductive complex [38, 41]. X-ray crystallographic studies of Topo I and Topo I-DNA complexes have revealed multiple interactions between DNA and Topo I in both the cleavable and the non-cleavable complex forms [42-44]. Pioneering studies of Horwitz and coworkers demonstrated that camptothecin could stabilize Topo I-DNA complexes throughout the cell cycle and that the stabilized complexes become toxic during S phase [45].

Table 2. Descriptors (DES) and Chemical Fragmentation Codes (CFC) Closely Correlated with the Inhibition of ABCG2-mediated MTX Transport

DES	CFC	Definition
H121	H121	One amine bonded to heterocyclic C
D023	D023	Substituents on a carbocyclic ring of a fused-ring heterocyclic system: Two C atoms of a fused carbocyclic ring bear substituents
M240	M240	Chain bonded to ring C
OH	H401	One –OH group
	H402	Two -OH groups
	H403	Three –OH groups
	H404	Four –OH groups
	H405	Five or more –OH groups
M531	M531	One carbocyclic system with at least one aromatic ring
J2	J2	Absence of ester (thioester)
H481	H481	One –OH group bonded to aliphatic C
RS4	D240	Three or more ring systems with O as the sole heteroatom
	D420	Three or more ring systems with one S atom as the sole heteroatom
	D430	Three or more ring systems with two or more S atoms as the sole heteroatoms
	D510	Three or more rings with O and S as the sole ring heteroatoms
	E300	Moephinan
	E310	Four-ring systems with one N atom as the sole heteroatom
	E320	Ergoline
	E330	Four-ring systems with 2 or more N atoms as the sole heteroatoms
	E540	Four-ring systems consisting solely of O, N, and C
	E870	Four-ring systems consisting solely of S, N, and C
	E920	Four or more rings with O, S, and N as heteroatoms
	G400	Four-ring system (at least one aromatic ring) with at lest one 3-, 4-, or 5-membered ring
	G410	Chrysene
	G420	Naphthacene
	G430	Other 6:6:6:6 systems
	G440	Other four-ring carboxylic systems
	G800	Four 6-membered alicyclic ring systems
	G810	other four 6-membered alicyclic ring systems except for G800 Steroids are not involved in G810

In the cases of OH and RS4, one descriptor (DES) represents multiple chemical fragmentation codes (CFC).

8. CPT-11 AND ABCG2-ASSOCIATED DRUG RESISTANCE

CPT-11 is one of the CPT-based anticancer drugs widely used in clinical practice today. CPT-11 *per se* is a pro-drug

Table 3. QSAR-based Prediction and Comparison with Observed Values in the Inhibition of ABCG2-mediated Methotrexate Transport

Test Compounds		Descriptor and Score								Inibition (%)		
		H121	D023	M240	OH	M531	J2	H481	RS4	Const.	Predicted	Observed
A-1	Dopamine	0	0	0	2	1	1	0	0	1	13.48	0
A-2	Epinephrine	0	0	0	3	1	1	1	0	1	-5.75	0
A-3	Norepinephrine	0	0	0	3	1	1	1	0	1	-5.75	0
A-4	GABA	0	0	0	0	0	1	0	0	1	1.74	0
A-5	Glutamic acid	0	0	0	0	0	1	0	0	1	1.74	0
A-6	Glycine	0	0	0	0	0	1	0	0	1	1.74	0
A-7	Histamine	0	0	0	0	0	1	0	0	1	1.74	0
A-8	Melatonin	0	0	0	0	0	1	0	0	1	1.74	4.3
A-9	Serotonin	0	0	0	1	0	1	0	0	1	14.14	0
B-1	Bepridil	0	0	0	0	0	1	0	0	1	1.74	20.7
B-2	Diltiazem	0	0	0	0	1	0	0	0	1	8.67	12.5
B-3	Fendiline	0	0	0	0	0	1	0	0	1	1.74	0
B-4	Nifedipine	0	0	1	0	1	0	0	0	1	30.64	28.6
B-5	Nicardipine	0	0	1	0	0	0	0	0	1	43.70	47.1
B-6	Prenylamine	0	0	0	0	0	1	0	0	1	1.74	0
B-7	Verapamil	0	0	0	0	0	1	0	0	1	1.74	11.6
C-1	Nicorandil	0	0	0	0	0	1	0	0	1	1.74	0
C-2	Pinacidil	0	0	0	0	0	1	0	0	1	1.74	0
E-1	Acetylsalicylic acid	0	0	0	0	1	0	0	0	1	8.67	0
E-2	Acemetacin	0	0	1	0	1	0	0	0	1	30.64	45.9
E-3	Indomethacin	0	0	1	0	1	1	0	0	1	10.65	0
E-4	Ibuprofen	0	0	1	0	1	1	0	0	1	10.65	9.6
E-5	Mepirizole	0	0	1	0	0	1	0	0	1	23.71	0
E-6	Naproxen	0	0	0	0	1	1	0	0	1	-11.32	0
F-2	Daunorubicin	1	0	1	4	1	1	0	1	1	33.99	43.6
F-3	Doxorubicin	1	0	1	5	1	1	1	1	1	14.75	12.9
F-4	Etoposide	0	1	1	3	1	1	0	1	1	6.31	15.8
F-5	5-Fluorouracil	0	0	0	0	0	1	0	0	1	1.74	0
F-6	Mitoxantrone	0	0	0	4	1	1	0	0	1	38.28	18.8
F-8	SN-38	0	0	1	2	0	1	0	0	1	48.51	62.3
G-1	Penicillin G	0	0	1	0	1	1	0	0	1	10.65	7.1
G-2	Novobiocin	0	1	1	2	1	1	0	0	1	85.55	90.1
H-1	Quinidine	0	0	1	1	0	1	1	0	1	4.48	0
H-2	*p*-Aminohippuric acid	0	0	0	0	1	1	0	0	1	-11.32	0
H-3	Reserpine	0	0	0	0	1	0	0	0	1	8.67	12.3

(Table 3. Contd….)

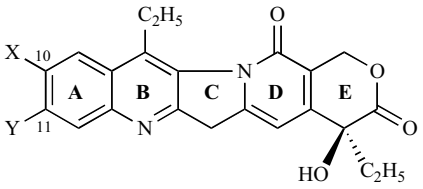
Test Compounds		Descriptor and Score								Inibition (%)		
		H121	D023	M240	OH	M531	J2	H481	RS4	Const.	Predicted	Observed
H-4	Prazosin	0	1	0	0	0	1	0	0	1	51.84	53.4
I-2	Hemin	0	0	1	0	0	1	0	0	1	23.71	20.1
I-3	Hematoporphyrin	0	0	1	2	0	1	0	0	1	76.64	89.2
I-4	Pheophorbide a	0	1	1	0	0	0	0	0	1	93.80	94.5
I-5	Quercetin	0	1	0	4	1	1	0	0	1	88.38	92.1

and undergoes carboxylesterase-mediated hydrolysis to form SN-38, a potent Topo I inhibitor [46, 47]. Despite the clinical effectiveness of CPT-11, acquired resistance to this anticancer drug has been reported. Hitherto, several mechanisms for the resistance to SN-38 and its analogues have been proposed: e.g., mutations or decreased expression of Topo I, ubiquitin/26S proteasome-mediated degradation of Topo I, increased expression of the UGT1A protein or single nucleotide polymorphisms (SNPs) of the *UGT1A* gene, increased activity of O^6-methylguanine-DNA-methyltransferase, a DNA repair enzyme, decreased activity of the carboxylesterase that catalyzes the biosynthesis of SN-38 from CPT-11 in the plasma and liver, and overexpression of drug export pumps (e.g., ABCC2/MRP2/cMOAT) [48-54]. About ten cell lines have so far been reported to be resistant to CPT-11 or SN-38. The resistant cell lines play an important role in elucidating the *in vivo* drug resistance mechanism, although results from experiments using resistant cell lines do not always accurately reflect the phenomenon.

Overexpression of ABCG2 has recently been shown to confer resistance to doxorubicin, mitoxantrone, and various CPT analogues. In our recent study [55], SN-38-resistant PC-6/SN2-5H2 human lung carcinoma cells were shown to overexpress ABCG2 with the reduced intracellular accumulation of SN-38. Plasma membrane vesicles prepared from those cells transported SN-38 in an ATP-dependent manner, confirming our idea that ABCG2 actively extrudes SN-38 from tumor cells and thereby confers drug resistance [25, 26].

9. NEW CAMPTOTHECIN ANALOGUES THAT CIRCUMVENT DRUG RESISTANCE

To circumvent SN-38 resistance, we tried to design new CPT-based lead compounds that are non-ABCG2 substrates. We have evaluated a total of fourteen CPT analogues that



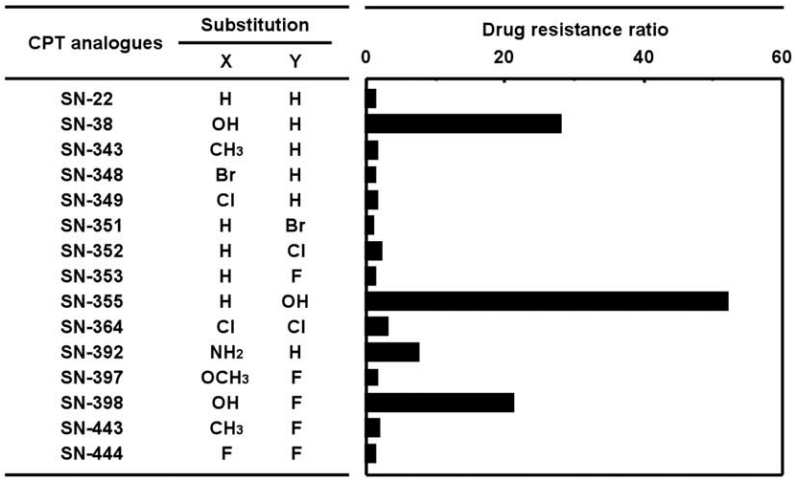


Fig. (3). Molecular structures of newly synthesized CPT analogues and their anticancer activity in ABCG2-transfected HEK293 (HEK293/ABCG2) cells. Drug sensitivity was determined by MTT assay after a 72-h drug exposure. Drug resistance ratios are calculated from the ratio of IC_{50} (HEK293/ABCG2)/IC_{50} (HEK293) where the IC_{50} value is the drug concentration representing a 50% reduction of cell growth.

were synthesized by replacing the hydroxyl group of SN-38 with others (hydrogen, halogenmethyl, methoxy groups, etc.) (Fig. (**3**)). While the lactone E ring is a prerequisite for the antitumor activity of CPT, modifications of the A or B rings do not significantly affect Topo I inhibition activity. In this context, the synthesized CPT analogues have various substitutions at positions 10 or 11 of the A ring, as demonstrated in (Fig. (**3**)). These CPT analogues are as potent as SN-38 with respect to Topo I inhibition in the cell-free system [55].

The drug resistance profile of those newly synthesized CPT analogues was examined by using both control and ABCG2-transfected HEK293 cells. Fig. (**3**) demonstrates drug resistance ratios for CPT analogues as determined by the MTT assay method. In the positive control with SN-38, ABCG2-transfected HEK293 cells were approximately 30-fold more resistant than the control HEK293 cells. As shown in Fig. (**3**), ABCG2-transfected HEK293 cells were resistant to SN-355, SN-392, and SN-398, but not to the other analogues. ABCG2-overexpressing membrane vesicles transported SN-38, SN-355, SN-392, and SN-398 in an ATP-dependent manner; however, other analogues such as SN-22, SN-343, SN-348, or SN-349, were not transported [56]. Based on these findings, it is speculated that those substrates of ABCG2, such as SN-38, SN-355, SN-392, and SN-398, have common properties in their molecular structures. SN-38 and SN-398 have one hydroxyl group at position 10, whereas SN-355 has the hydroxyl group at position 11 (Fig. (**3**)). In addition, SN-392 has one amino group at position 10. Hydroxyl and amino groups are considered to be important for the formation of hydrogen bonds. Interestingly, the other CPT analogues do not have such groups at positions 10 or 11 (Fig. (**3**)). It is likely that hydrogen bond formation is involved in substrate recognition and/or the transport processes of ABCG2. In addition, the planar structure of the fused heterocyclic rings with conjugated π-orbits is considered to be critical for the interaction with the active site of the ABCG2 protein.

10. MOLECULAR ORBITAL CALCULATIONS AND NEURAL NETWORK QSAR ANALYSIS

To further develop a platform for the molecular modeling of anticancer drugs to circumvent ABCG2-associated drug resistance, we have carried out quantum chemical calculations and neural network quantitative structure-activity relationship (QSAR) analysis. Firstly, we have applied the neural network analysis to estimate the hydrophilic properties of CPT analogues, since hydrogen bond formation is supposed to be critically involved in ABCG2-associated transport. The initial structures of CPT analogues were generated by molecular mechanics procedures and semi-empirical MO calculations using AM1 parameters [57]. Thereafter, the solvation free energy (ΔG) and the electrostatic potential (ESP) were evaluated by molecular orbital (MO) calculations. To calculate ΔG values, the solvation effects of water simulated by COSMO [58] were additionally employed. ΔG was defined as $\Delta G = E_{COSMO} - E_{in\ vacuo} + SASA*0.00542 + 0.92$, where E_{COSMO}, $E_{in\ vacuo}$, and SASA are total energies of the SN-38 analogues calculated by AM1/COSMO, AM1/in vacuo, and solvation accessible surface area, respectively [59]. When drug resistance ratios were plotted versus the ΔG values, CPT analogues were classified into two distinct groups, namely, the substrate and the non-substrate groups for ABCG2 (data not shown).

To further investigate the electrostatic properties of CPT analogues, ESP iso-surfaces (±0.01 atomic unit) were generated by *ab initio* MO calculations by using the restricted Hartree-Fock method with the MIDI-4 plus polarization function [60], where one atomic unit corresponds to 1.6022E-19 coulomb. As demonstrated in (Fig. (**3**)), SN-38 and SN-398 that are classified as the substrate group have a negative potential area (indicated by an arrow) at position 10 in the A ring. On the other hand, SN-22 does not exhibit such a negative potential area (Fig. (**4**)). From these data, the negative potential area at position 10 or 11 in the A ring is critical for exerting CPT analogues hydrophilic properties as well as for facilitating the hydrogen bond formation with the active site of ABCG2. Thus, MO calculation-based neural network QSAR analysis may provide a useful approach to understand the substrate specificity of ABCG2 and also to design new anticancer drugs that circumvent ABCG2-associated drug resistance.

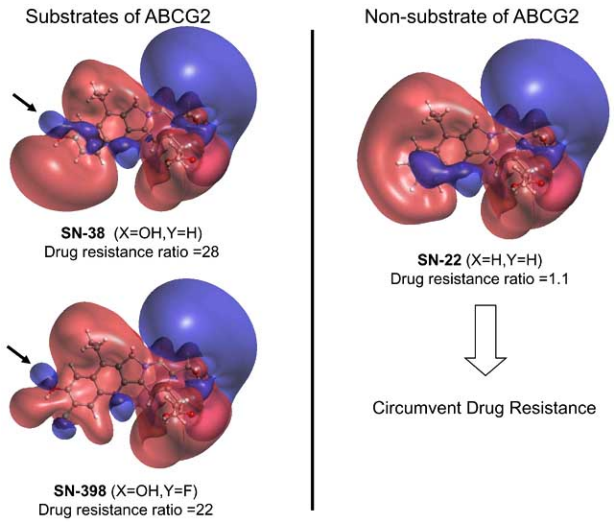


Fig. (4). Electrostatic potential (ESP) maps of SN-22, SN-38, and SN-398. ESP iso-surfaces (± 0.01 atomic unit) were generated by *ab initio* MO calculations using restricted Hartree-Fock (RHF) method with the MIDI-4 plus polarization function. 1 atomic unit = 1.6022E-19 coulomb.

11. CONCLUDING REMARKS

Inhibitors of ABCG2 are of interest as chemosensitizers for clinical drug resistance and for improving the pharmacokinetics of poorly absorbed chemotherapeutic drugs. There are increasing numbers of reports on ABCG2 inhibitors [61-70]. Several laboratories have provided evidence that tyrosine kinase inhibitors, such as gefitinib (Iressa; ZD1839) and imatinib (Gleevec; STI571) inhibit ABCG2 function. Protein kinases are potential drug targets for treatment of a variety of diseases, including cancer. In particular, specific tyrosine kinase inhibitors are rapidly being developed as new drugs for the inhibition of malignant cell growth and metastasis formation.

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ABBREVIATIONS

ABC	=	ATP-binding cassette
CFC	=	Chemical fragmentation code
CPT	=	Camptothecin
DES	=	Descriptor
E_3S	=	Estrone-3-sulfate
HEK	=	Human embryonic kidney
MO	=	Molecular orbital
MTT	=	3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
MTX	=	Methotrexate
NSAIDs	=	Non-steroidal anti-inflammatory drugs
QSAR	=	Quantitative structure-Activity relationship
SLC	=	Solute carrier
Topo I	=	DNA topoisomerase I
UGT1A	=	UDP-glucuronosyl transferase 1A

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