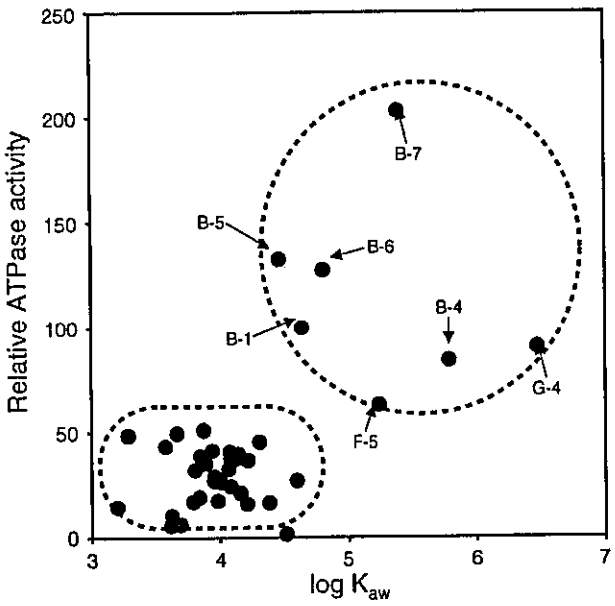


Fig. 5. Relationship between the ABCB1 ATPase activities and the $K_{aw}$ values of the therapeutic drugs and compounds tested. The ATPase activities are expressed as relative values as compared with the activity measured with 10 $\mu$M verapamil (100%). (From ref. 71)

$$\Gamma^* = (1/RT)d\pi/dlnC \qquad \text{Equation (2).}$$

Integral forms of Equation (1) can also be given. Particularly useful for our purpose is the Szyszkowski equation [20] which may be written as:

$$\pi = RT\Gamma^* \ ln\ (1 + K_{aw}C) \qquad \text{Equation (3),}$$

where $K_{aw}$ is the air/water partition coefficient. By fitting Equation (3) to the measured $\pi/C$ curve by using $\Gamma^*$ determined according to Equation (2), the air/water partition coefficient, $K_{aw}$, was evaluated. Seelig and Landwojtowicz have demonstrated that the $K_{aw}$ value is closely related to the Km values of ABCB1 substrates.[77] Their experimental results strongly suggest that the $K_{aw}$ value is a useful indicator for the prediction of ABCB1 substrates, even better than logP values.[77]

We have measured the surface activity of those 41 different compounds. **Figure 5** demonstrates the relationship between the ABCB1 ATPase activities and the $K_{aw}$ values of the therapeutic drugs and compounds tested. The ABCB1 ATPase activities are the same as the results presented in **Fig. 4**. The two-dimensional plot of log $K_{aw}$ values vs. ABCB1 ATPase activities (**Fig. 5**) revealed that test compounds could be clearly divided into two groups, namely, ABCB1 substrate and non-substrate groups that are indicated by circles in the figure. Compounds with log $K_{aw}$ values higher than 4.3 could be regarded as candidate substrates for ABCB1. These results suggest that ABCB1 substrates are surface-active and can be readily dissolved in the lipid bi-layer of cellular membranes.

**SAR analysis for the substrate specificity of ABCB1:** To gain more insight into the relationship between the molecular structure of compounds and the ABCB1 ATPase activity, we have performed an SAR analysis. Up to now, several research groups have intensively investigated the SAR of ABCB1 substrates[78-82] and tried to establish theoretical calculation methods, such as MolSurf parametrization and PLS statistics.[83] Recently, we have developed a different approach for the SAR analysis to gain insight into the substrate specificity of ABCB1. Namely, we used the chemical fragmentation codes to describe the chemical structures of a variety of substrates and non-substrates for ABCB1. Derwent Information, Ltd., developed this structure-indexing language suitable for describing chemical patents. The chemical fragmentation codes were originally created in the early 1960's in answer to the need for accessing the increasing number of chemical patents. Markush TOPFRAG is the software that generates the chemical fragment codes from chemical structure information.[84,85]

As described above, we have first measured ABCB1 ATPase activity toward a total of 41 different drugs and compounds by using our high-speed screening system. The Markush TOPFRAG was then used to generate chemical fragmentation codes for each compound tested. **Table 3** exemplifies the chemical fragmentation codes describing the molecular structure of verapamil (B-1). In this way, however, steroids (group C) were excluded from this analysis, because the Markush TOPFRAG program does not have an algorithm to generate chemical fragmentation codes for steroids.

The multiple linear regression analysis was the carried out to gain a relationship between the ABCB1 ATPase activity and the chemical fragmentation codes thus generated. Thereby we could identify several sets of chemical fragmentation codes related to the substrate specificity of ABCB1. A total of six best-fitting models were created (**Fig. 6**), where the predicted activity of the ABCB1 ATPase was well correlated with the observed ATPase activity. **Table 4** summarizes the contents of those multiple linear regression analysis models, and **Table 5** provides explanations for chemical fragmentation codes generated in the analysis. These results demonstrate that the moieties represented by the chemical fragmentation codes of J581, G100, and M331 positively contributed to the ATPase activity, whereas those of M531 and F014 had negative contributions. Among those chemical fragmentation codes, J581 had the greatest contribution (**Table 4**), suggesting that an oxo group bonded to an aliphatic carbon (**Table 5**) is an important moiety for the recognition and/or transport by the ABCB1 protein. In addition, it is suggested that unfused aromatic ring(s) and straight carbon chain(s)

**Table 3.** Chemical fragmentation codes describing the molecule structure of verapamil

**Verapamil**

S (G100(P)H181(P)H543(P)M333(P)M414(P)M532(P)("L140" OR "L145"))/M0,M2,M3,M4
S L1(P)(M210(P)M283(P)M312(P)M316(P)M321(P)M332(P)M342(P)M343)/M2,M3,M4
S L2(P)((M370(P)M392) OR (M371(P)M373(P)M391))/M2,M3,M4
S L3(P)(M270 OR (M272(P)M273(P)M281))/M2,M3,M4
S L4(P)(G015(P)G019(P)H103(P)M211)/M2,M3,M4
S (L1(P)M900/M0) OR (L1(P)M901/M2,M3,M4) OR (L4(P)M902/M2,M3,M4)
S L6 OR L5
S L7(NOTP)(H2 OR H3 OR H4 OR H6 OR H7 OR H9 OR J0 OR J1 OR J2 OR J3)/M2,M3,M4
S L8(NOTP)(J4 OR J5 OR J6 OR J9 OR K1 OR K2 OR K3 OR K4 OR K5 OR K6)/M2,M3,M4
S L9(NOTP)(K7 OR K8 OR K9 OR "L2" OR "L3" OR "L4" OR "L5" OR "L6" OR "L7")/M2,M3,M4
S L10(NOTP)("L8" OR "L9" OR M1)/M2,M3,M4

The chemical fragmentation codes were generated by using the Markush TOPFRAG program.

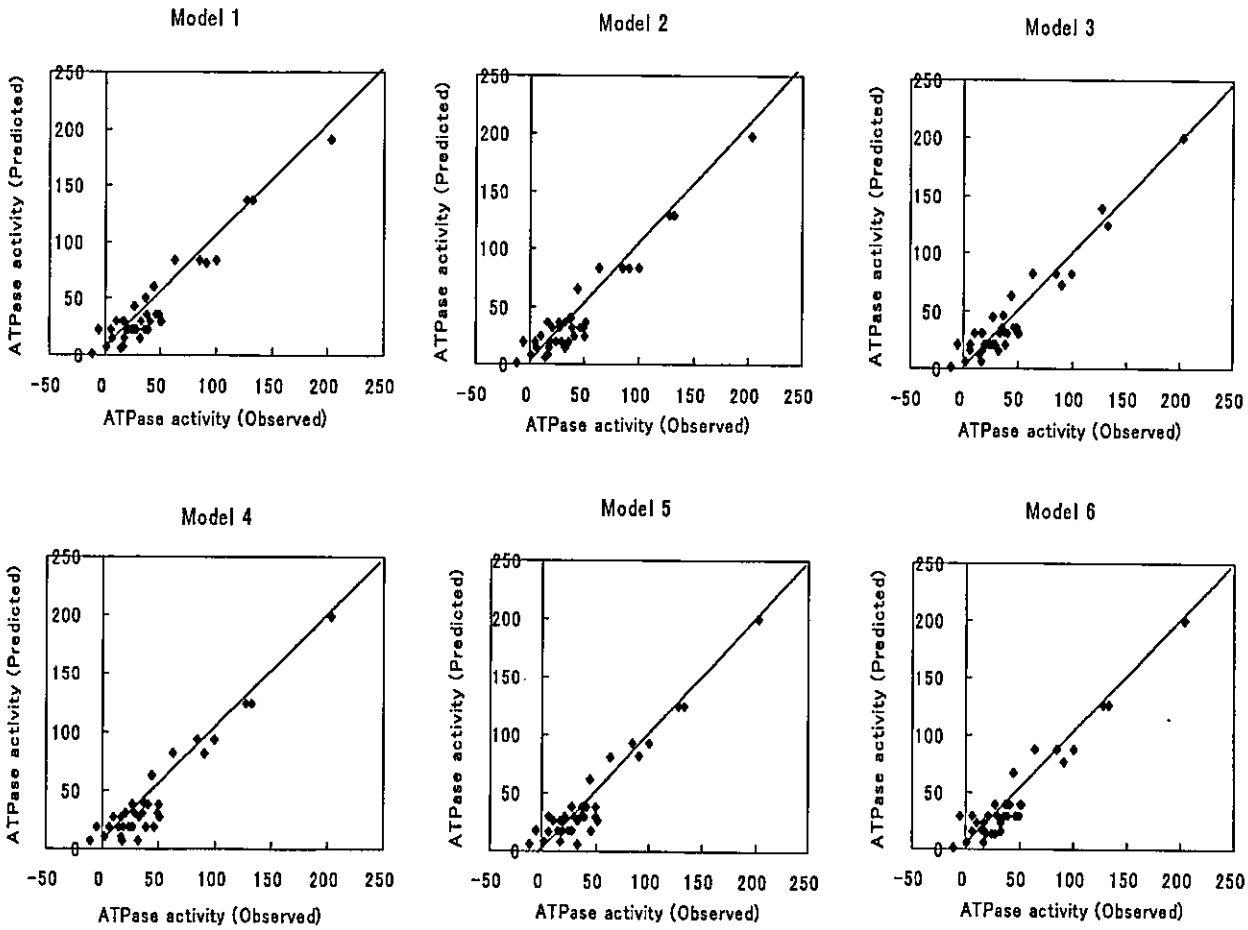


**Fig. 6.** Relationships between the relative ATPase activities observed in Fig. 4 and the ATPase activities predicted from the multiple linear regression analysis. (From ref. 71)

are important chemical moieties for the substrate specificity of ABCB1.

The uniqueness of this approach resides in the facts that ABCB1 ATPase activity is described as a linear combination of chemical fragmentation codes and that the coefficient for each chemical fragment code reflects the extent of the contribution of a specific chemical moiety to the ATPase activity. The point in the catalytic cycle at which substrate-binding takes place, and details of how ATP hydrolysis drives transport may be critical for understanding the mechanism of substrate specificity[86]. It would be of importance to further expand this

**Table 4.** Multiple linear regression analysis models to predict ABCB1 ATPase activity toward tested compounds

| Chemical Fragmentation Code | Model 1 | Model 2 | Model 3 | Model 4 | Model 5 | Model 6 |
|---|---|---|---|---|---|---|
| J581 | 96.87 | 90.10 | 90.67 | 82.86 | 84.69 | 97.04 |
| G100 | 54.47 | 59.33 | 51.62 | 55.01 | 54.80 | 49.17 |
| M331 | 38.42 | 46.44 | 42.30 | 42.91 | 43.67 | 37.71 |
| M270 | 0 | 0 | 0 | 0 | 11.61 | 0 |
| M272 | 0 | 0 | 0 | 11.40 | 0 | 0 |
| M531 | −61.63 | −64.51 | −61.04 | −62.64 | −63.38 | −59.43 |
| F014 | −28.31 | −22.44 | −29.09 | −19.80 | −20.92 | −22.41 |
| H100 | 0 | 0 | 0 | 0 | 0 | −15.64 |
| M321 | 0 | 0 | −14.60 | 0 | 0 | 0 |
| M370 | 0 | −12.56 | 0 | 0 | 0 | 0 |
| M391 | −14.29 | 0 | 0 | 0 | 0 | 0 |
| Constant | 43.44 | 36.61 | 45.01 | 26.99 | 26.40 | 38.79 |
| R= | 0.953 | 0.952 | 0.953 | 0.952 | 0.952 | 0.954 |

The ABCB1 ATPase activity is formulated as a linear combination of chemical fragmentation codes weighted by the corresponding coefficient, where the symbol of "*i*" in the parentheses designates a specific chemical fragmentation code.
ABCB1 ATPase activity (Predicted) = $\Sigma C(i) \times$ Chem. Frag. Code ($i$) + Constant. R: Correlation coefficient.

**Table 5.** Explanation for the chemical fragmentation codes used for the prediction of the ABCB1 ATPase activity

| Chemical Fragmentation Code | | Ext. Code | Explanation |
|---|---|---|---|
| J58 | Oxo group bonded to aliphatic C | J581 | One oxo group bonded to aliphatic |
| G1 | Unfused aromatic rings | G100 | Unfused aromatic ring(s) present, no other carbocyclic ring systems are present |
| M33 | Straight or branched carbon chains | M331 | Straight Carbon chain with $-CH_3$, $-C=CH_2$, and/or $-C\equiv CH$ |
| M27 | Chain bonded to U | M270 | Chain bonded to U |
| M53 | Carbocyclic systems with at least one aromatic ring | M272 | Chain bonded to O |
| F01 | Positions substituted | M531 | One M53 code |
| H10 | Type of amine | F014 | Position 4 substituted |
| M32 | Multipliers for Subset M31 | H100 | One primary amine |
| | M31:Number of C atoms in polyvalent chain | G321 | One or more M31 code used once |
| M37 | Carbon chain bonded to ring C and (U and/or C=U and/or C≡CH) but not V, C=V, C≡V | M370 | Carbon chain bonded to ring C and (U and/or C=U and/or C≡CH) but not V, C=V, C≡V |
| M39 | Multipliers for codes M350 to M383 (polyvalent carbon chain attachments) | M391 | One or more of codes used once |

U = C,H,O,S,Se,Te or N
V = atom other than U

analysis with a large number of structurally diverse compounds.

## Conclusion

**Application of SAR analysis to functional evaluation of genetic polymorphisms:** The present review conveys a new strategy of efficiently analyzing the relationship between the substrate specificity of ABCB1 and the chemical structure of substrates. This approach is applicable for the functional analysis of genetic polymorphisms of ABCB1. The effect of SNPs on the transport activity may depend on substrates tested, and therefore the functional analysis of SNPs using a wide variety of substrates is of great interest. One amino acid substitution can alter interactions between the active site of ABCB1 and substrate molecules. Therefore, it is critically important to quantitatively analyze and evaluate such structure-related interactions. In this context, the new SAR analysis using chemical fragmentation codes will provide a powerful tool to quantify the impact of genetic polymorphisms on the function of ABCB1.

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*Journal of Experimental Therapeutics and Oncology*, Vol. 4, pp. 239-246
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# Human ATP-binding cassette transporter ABCC10: expression profile and p53-dependent upregulation

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*(Received August 26, 2004; accepted September 1; Sponsored by Toshihisa Ishikawa.)*

Human ATP-binding cassette (ABC) transporter genes are classified into seven sub-families, where "C" subfamily comprises a total of 13 gene members. The ABCC10 cDNA was cloned in the human full-length cDNA project at the Kazusa DNA Research Institute. However, current information is limited regarding its physiological function and gene expression. In the present study, we have investigated the expression of the *ABCC10* gene to gain insight into its biological nature. By quantitative PCR, *ABCC10* gene expression is demonstrated to be highest in pancreas among the adult and fetal tissues and tumors presently tested. Decreased expression was observed when resting T- and B-cells were activated. Furthermore, when we examined its expression under apoptotic conditions, we found that ABCC10 mRNA levels remarkably increased in doxorubicin-treated MCF7 cells, whereas its up-regulation was suppressed in p53-dominant-negative MCF7 cells. These results suggest that expression of the *ABCC10* gene is regulated in a p53-dependent manner during DNA-damage-related apoptosis.

**Keywords:** ABC transporter; ABCC10, multidrug resistance-associated protein; p53; apoptosis; doxorubicin; quantitative PCR

**Abbreviations:** ABC, ATP-binding cassette transporter; MRP, multidrug resistance-associated protein; ORF, open reading frame; TNF, tumor necrosis factor; DFO, desferrioxamine.

## INTRODUCTION

The ATP-binding cassette (ABC) transporters form one of the largest protein families and play biologically important roles as membrane transporters or ion channel modulators. According to the recently published sequence of the human genome, more than 50 human ABC transporter genes (including pseudogenes) are anticipated to exist in the human genome. Hitherto 48 human ABC-transporter genes have been identified and sequenced (recent reviews: 1-4). Mutations in the human ABC transporter genes have been reported to cause such genetic diseases as Tangier disease, cystic fibrosis, Dubin-Johnson syndrome, Stargardt disease, and sitosterolemia (2,3). Based on the arrangement of their molecular structural components, i.e., the nucleotide binding domain and the topology of transmembrane spanning domains, human ABC transporters are classified into seven different gene families designated as A to G (the new nomenclature of human ABC transporter genes: http://gene.ucl.ac.uk/nomenclature/genefamily/abc.html).

The "C" subfamily includes a total of 13 members. ABCC1 (MRP1) was first identified by Cole et al. in the molecular cloning of cDNA from human multidrug-resistant lung cancer cells (4). After the discovery of the ABCC1 (MRP1) gene, six human homologues, ABCC2 (cMOAT/MRP2), ABCC3

(MRP3), ABCC4 (MRP4), ABCC5 (MRP5), ABCC6 (MRP6), and ABCC10 (MRP7) have been successively identified. Those ABC transporters exhibit a wide spectrum of biological functions and are involved in the transport of drugs as well as endogenous substances (5-10). Furthermore, the CFTR (ABCC7) gene encodes the cystic fibrosis transmembrane conductance regulator that functions as a chloride ion channel. It is also well documented that mutations of this gene cause cystic fibrosis, a genetic disease (11). ABCC8 and ABCC9 are sulphonylurea receptors, SUR1 and SUR2, and they play a critical role in the regulation of glucose-stimulated insulin secretion (12).

Recently, our group and others have identified two novel genes, *ABCC11* and *ABCC12*, which are tandemly located on chromosome 16q12.1 (13-16). The deduced ABCC11 protein shows high homology with ABCC5. Furthermore, our group has identified the *ABCC13* gene (17). This gene, consisting of 14 exons, is located on chromosome 21q11.2. The *ABCC13* gene exhibits a unique sequence, which lacks the Walker A motif. The ABCC13 gene is highly expressed in hematopoietic systems, such as fetal liver and bone marrow.

The *ABCC10* gene (FLJ00002) has been identified at the Kazusa DNA Research Institute in the human large cDNA sequencing project. The deduced ABCC10 protein has 17 trans-membrane domains and two ATP-binding cassettes (18). Its amino acid homology with the other members of the "C" subfamily is in the range of 25 to 35%, which is relatively low. However, it has not yet been elucidated whether ABCC10 functions as a transporter or an ion channel regulator. In the present study, we have characterized ABCC10 in terms of its expression profile assessed by quantitative PCR. We have found that the expression of ABCC10 is related to doxorubicin-induced apoptosis in MCF7 cells. In particular, expression of the *ABCC10* gene is suggested to be regulated by p53 or its signaling pathway(s) during DNA-damage induced apoptosis.

## MATERIALS AND METHODS

### *Human cDNA panels*

Human Multiple-Tissue cDNA (MTC) Panels and Apoptosis cDNA Panels were purchased from Clontech (Palo Alto, CA, USA) for the analysis of tissue distribution and gene expression under apoptotic conditions, respectively.

### *Cell Culture and Induction of Apoptosis*

The human chronic myelogenous leukemia K562 cell line was cultured in RPMI1640 medium (Invitrogen Corp., Carlsbad, CA) in a humidified chamber (37°C, 5% $CO_2$). RPMI1640 medium was supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml). The number of living cells was determined in a hemocytometer by trypan blue dye exclusion. To induce apoptosis, cells (5 x $10^5$ cells/ml) were treated with doxorubicin at different concentrations (see Results and Discussion for details).

### *Detection of DNA Fragmentation*

Drug-treated cells were harvested, washed once with PBS(-), and lysed with 100 µl of cell lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM EDTA (pH 8.0), 0.5% Triton X-100). The cell lysate was centrifuged at 15,000 rpm at 4°C for 5 min, and the resulting supernatant including fragmented-DNA was transferred into a new tube. After treatments with RNaseA (QIAGEN, Hilden, Germany) at 37°C for 60 min and with proteinase K (Roche Diagnostics, Indianapolis, IN, USA) at 50°C for 30 min, the sample was mixed with 20 µl of 5 M NaCl and 120 µl of isopropanol and then centrifuged at 15,000 rpm at 4°C for 30 min. The resulting precipitate (containing DNA) was dissolved in 20 µl of TE buffer and separated on a 2%-agarose gel. Fragmented DNA was stained with ethidium bromide and detected by UV irradiation.

### *RNA Extraction and First-Strand cDNA Synthesis*

RNA was extracted from cultured cells (i.e., K562 cells) by using ISOGEN (WAKO Pure Chemical Industries, Japan) according to the manufacturer's instruction. cDNA was synthesized from 2 µg of total RNA by using the SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA) with oligo(dT) primers. The synthesized cDNA was diluted with $ddH_2O$ to have appropriate concentrations for PCR.

### *Quantitative PCR*

Quantitative PCR analysis was performed with TaKaRa Ex Taq™ R-PCR ver. in a TaKaRa Smart Cycler™ System (TaKaRa, Japan), where amplification of DNA was monitored with SYBR Green I. The reaction mixture contained 2 µl of cDNA, 2.5 µl of 10 x PCR buffer, 0.75 µl of 10 mM dNTP mix, 0.75 µl of 10 µM PCR primers, 0.3 µl of 250 mM $Mg^{2+}$ solution, 0.25 µl of 1/300-dilluted SYBR Green I (Bio-Whittaker Molecular Applications, Rockland, ME, USA), and 0.25 µl of Ex Taq polymerase in a total volume of 25 µl.

The following PCR primers were used to determine the copy number of ABCC10: the sense primer CGGGCCTGGTGAGCAGCTTCACACA (q-C10F) and the antisense primer CGGTACGCCAACA-CCACGTCCTGGAACT (q-C10B). The PCR

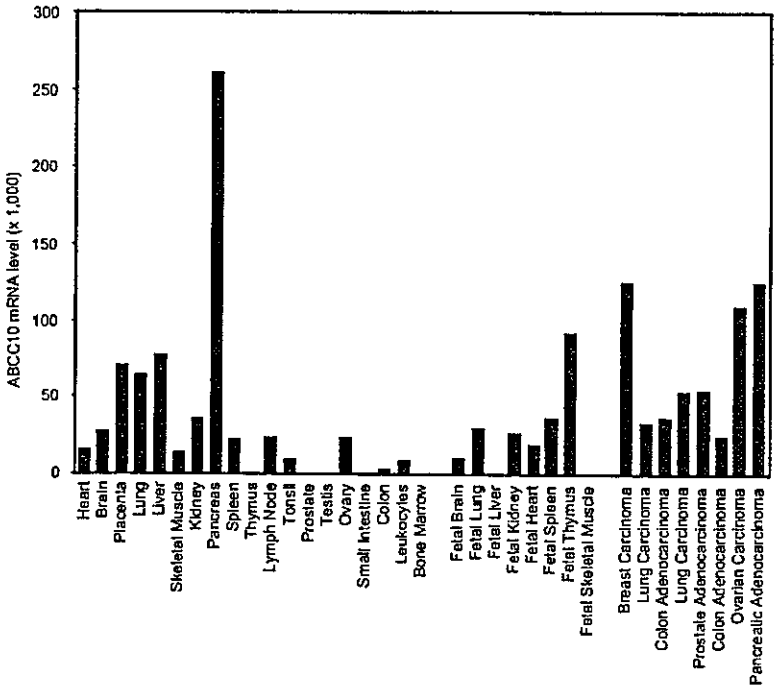


***Figure 1.*** Tissue Distribution of ABCC10. Levels of ABCC10 mRNA in human adult and fetal tissues and tumors were determined by quantitative PCR (see Materials and Methods). The Human MTC Panels I and II as well as Immune System, Fetal, and Tumor MTC Panels (Clontech) were used as PCR templates.

conditions were as follows: 95°C for 30 s, 45 cycles of 95 °C for 15 s, 70°C for 20 s, and 91°C for 7 s. The size of DNA, thus amplified, was 172-bp, which corresponds to nucleotides 3596-3767 in the ORF of ABCC10 cDNA.

The copy number of ABCC10 was normalized with that of β-actin. For this purpose we have used the following β-actin-specific PCR primers: the sense primer TGAAGTACCCCATCGAGCACG (q-actinF) and the antisense primer CAAACATGATCT-GGGTCATCTTCTC (q-actinB). PCR conditions were: 95°C for 30 s, 40 cycles of 95°C for 15 s, 58°C for 15 s, 72°C for 15 s, and 90°C for 7 s. The amplified DNA was 174-bp in size.

## RESULTS AND DISCUSSION

### *Expression of ABCC10 in adult and fetal tissues*

Human ABCC10 has recently been described as a member of the "C" sub-family of ABC transporters. Its cDNA was cloned in the human full-length cDNA project at the Kazusa DNA Research Institute (19,20). At the present time, however, little is known about the physiological function of ABCC10. In this study, we used the human Multiple-Tissue cDNA Panels (see Materials and Methods) to analyze the tissue-specific expression of ABCC10 in human adult and fetal tissues and tumors by means of the quantitative PCR method. Among all tissues examined, the highest expression level of ABCC10 mRNA was observed in the adult pancreas, followed by that in liver, placenta, and lung (Figure 1). In contrast, ABCC10 mRNA was not detected in testis, small intestine, or bone marrow under the present PCR conditions.

The heart exhibited a low, but detectable level of ABCC10 mRNA (Figure 1). As summarized in Table 1, its mRNA levels at various sites in the cardiovascular system suggest a heterogeneous distribution of ABCC10. The highest expression levels were observed in the auricle, dextra (10,270 copies) and atrium, right (10,266 copies), whereas the lowest was in the aorta (1,325 copies).

In fetal tissues, the expression profile of ABCC10 was different from that in the adult (Figure 1). Whereas ABCC10 was broadly expressed in all tissues here tested, the highest expression was observed in the fetal thymus. In addition, the level of ABCC10 mRNA in the fetal liver was much lower than that in the adult liver.

In human tumors, ABCC10 mRNA was detected in

***Table 1.*** Levels of ABCC10 mRNA in the cardiovascular system.

| Site | mRNA level |
|---|---|
| Aorta | 1,325 |
| Apex of the Heart | 8,637 |
| Atrium, left | 8,206 |
| Atrium, right | 10,266 |
| Auricle, dextra | 10,270 |
| Auricle, sinistra | 7,196 |
| Ventricle, left | 2,177 |
| Ventricle, right | 4,905 |
| Interventricular septum | 9,772 |
| Atrioventricular node | 7,286 |

Levels of ABCC10 mRNA within the human cardiovascular system were determined by quantitative PCR. The Human Cardiovascular System MTC Panel was used as the PCR template.

all of the samples examined (Figure 1). In particular, expression levels were notably high in breast carcinoma (GI-101), ovarian carcinoma (GI-102), and pancreatic adenocarcinoma (GI-103). These results suggest that the *ABCC10* gene is nearly ubiquitously expressed in both normal and cancer tissues.

***Expression of ABCC10 in peripheral blood fractions***

Quantitative analysis of ABCC10 expression in peripheral blood fractions was performed in a similar fashion (Figure 2). The levels of ABCC10 expressed in activated CD8+ cells (T-suppressor/cytotoxic) and CD19+ cells (B-cells) were one sixth and one ninth of those in resting CD8+ and CD19+ cells, respectively. These findings indicate that the expression of the *ABCC10* gene in certain cell types is down-regulated through the immune response.

In contrast to the findings for CD8+ and CD19+ cells, the levels of ABCC10 expressed in CD4+ cells (T-helper/inducer) was not significantly affected by activation (Figure 2). Levels of ABCC10 mRNA in mononuclear cells including T- and B-cells and monocytes apparently decreased; however, this reduction was not statistically significant under the criterion of $P<0.05$. Moreover, about 20,000 copies of the ABCC10 transcript were detected in CD14+ cells (monocytes) (data not shown). At present, the physiological function of ABCC10 in peripheral blood cells is not known. It would be of great interest to characterize the regulation of the *ABCC10* gene expression under immune responses.

***Expression profiles of ABCC10 in relation to apoptosis***

Relevance of the relationship between the drug response of cancer cells and the expression of ABC-transporter genes has been widely investigated (21). Certain ABC-transporters, such as ABCB1, ABCC1, and ABCG2, are known to play a pivotal role in the multidrug-resistance of cancer cells to cytotoxic antitumor drugs. Accumulating evidence suggests that p53 tumor suppressor is involved in the regulation of *ABCB1* and *ABCC1* gene expression (22-24). Hitherto it has been well documented that such anti-tumor drugs as doxorubicin, cisplatin, and vincristine induce apoptosis. The breast carcinoma MCF7 cell line is widely used as a model for p53-dependent apoptosis, because the cell line expresses high levels of the wild-type p53 protein (25-27).

In the present study, we have investigated the drug-response of the *ABCC10* gene in doxorubicin-treated MCF7 cells by quantitative PCR (Figure 3A). It is noteworthy that the level of ABCC10 mRNA was enhanced 54-fold by doxorubicin-induced apoptosis in wild-type MCF7 cells, but only 4.7-fold in p53 dominant-negative MCF7 cells. The dominant-negative MCF7 cells contain a plasmid that expresses mutant p53 derived from MDD2 cells, and that functionally inactivates wild-type p53. These results suggest that doxorubicin-stimuli lead to the expression of ABCC10 and that this induction is mediated by a p53-dependent process.

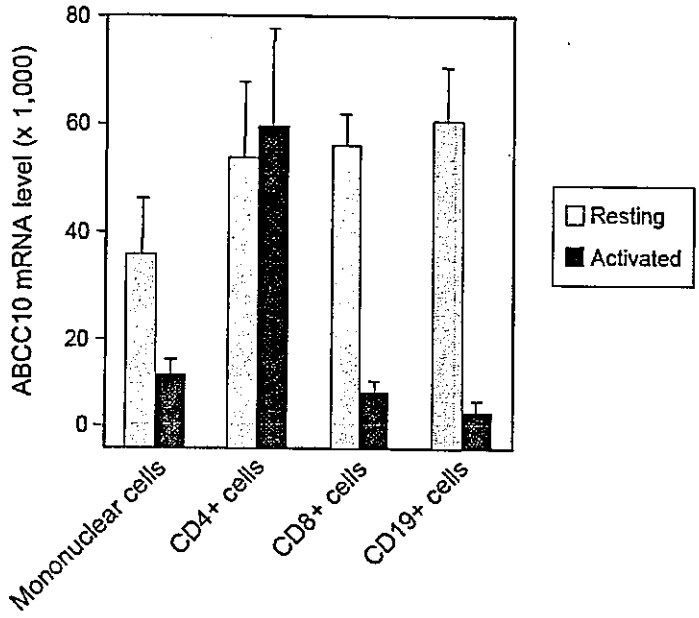


***Figure 2.*** Levels of ABCC10 mRNA in peripheral blood fractions. ABCC10 mRNA levels in mononuclear cells (B- and T-cells and monocytes), CD4+ cells (T-helper/inducer), CD8+ cells (T-suppressor/cytotoxic) and CD19+ cells (B-cells) were determined by quantitative PCR. The Blood Fraction Panel was used as PCR template. Gray and black bars indicate resting and activated cells, respectively. Results are shown as means ± S.D. of triplicate experiments. Asterisk (*) indicates significant difference as assessed by Student's *t*-test (P † 0.05).

DNA can be damaged by doxorubicin via inhibition of topoisomerase II, and those damage signals are considered to activate wild-type p53. Once activated, p53 can initiate the transcription and subsequent expression of various downstream genes that commit the cell to cellular arrest and/or apoptosis. The induction of ABCC10 expression observed in this study may be regulated by wild-type p53 or its downstream-signals.

There are several pathways that can lead to apoptosis, such as anticancer drug treatment, death-receptor mediated signaling, iron chelator treatment, heat-shock, and UV irradiation. To examine the contribution of p53-mediated pathways to the apoptosis-related induction of ABCC10 gene expression, we have studied the effects of TNFα– and DFO-induced apoptosis signals on the induction of ABCC10. As demonstrated in Fig 3B, TNFα-treatment had no significant effects on the expression levels of ABCC10, whereas DFO-treatment transiently enhanced the level of ABCC10 mRNA in HeLa cells. A 2.7-fold increase was observed in the ABCC10 mRNA levels at 6 h after the onset of the DFO-treatment. Although signaling pathways in DFO-induced apoptosis are not well characterized, DFO reportedly stabilizes the p53 protein and/or activates hypoxia-inducible factor 1α (HIF-1α) (25) and caspases (28). On the other hand, TNFα binds to the TNF receptor (TNFR) to activate the TNFR-associated death domain (TRADD), and this activation leads to apoptosis through the Fas-associated death domain (FADD) (29). It is important to note that the TNFα-mediated apoptosis pathway does not involve p53-associated steps, which is consistent with our finding that TNF did not induce ABCC10 during apoptosis.

To further examine the potential involvement of the p53-mediated pathway in ABCC10 induction, we have treated K562 cells with 400 ng/ml doxorubicin and measured the ABCC10 expression levels. Doxorubicin-induced apoptosis was determined by fragmentation of genomic DNA (Figure 3C). Nonetheless, the level of ABCC10 mRNA was little changed during the treatment (Figure 3D). Since K562 cells have a mutated p53 gene (30,31), this result indirectly supports our finding that dominant-negative p53 suppresses the induction of ABCC10 during the

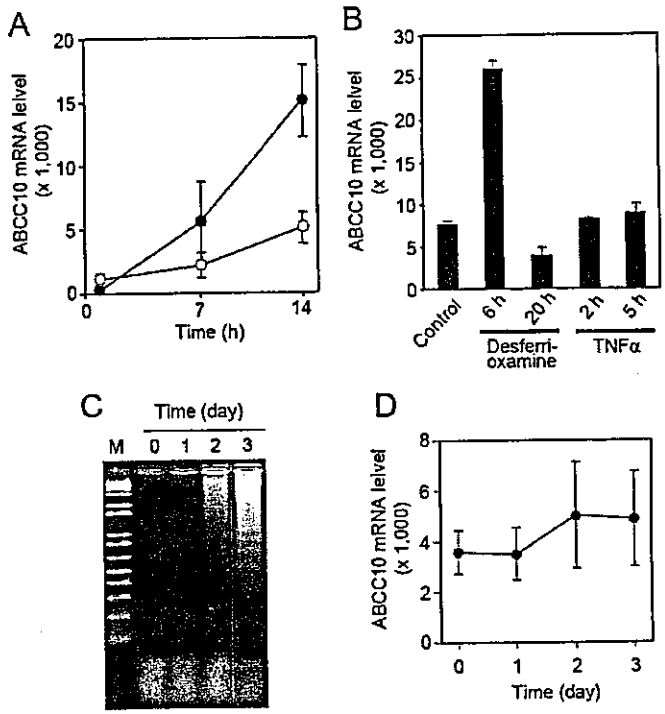


***Figure 3.*** Expression of ABCC10 under apoptotic conditions. (A) Time courses of ABCC10 mRNA levels in MCF7 cells treated with doxorubicin. MCF7 cells (closed circles) and p53 dominant-negative MCF7 cells (open circles) were treated with doxorubicin at the final concentration of 400 ng/ml and cultured for the indicated hours. (B) ABCC10 mRNA levels in HeLa cells treated with 0.05 ng/ml TNFα or 250 μM DFO (desferrioxamine). HeLa cells were treated with TNFa and DFO at the final concentration of 0.05 ng/ml and 250 μM, respectively, and cultured for the indicated hours. (C and D) DNA fragmentation and ABCC10 mRNA levels in K562 cells treated with doxorubicin. K562 cells were treated with doxorubicin at the final concentration of 400 ng/ml and cultured for the indicated days (see Materials and Methods). DNA fragmentation was detected by agarose gel (2%) electrophoresis (C). Levels of ABCC10 mRNA were determined by quantitative PCR and normalized with β-actin mRNA levels (D).

apoptotic process induced by doxorubicin. Taken together, the expression of ABCC10 is up-regulated by doxorubicin, and it is strongly suggested that induction of the *ABCC10* gene is regulated by p53 or its signaling pathways during DNA-damage-related apoptosis in MCF7 cells.

## CONCLUSION

Chen et al. have recently reported that human ABCC10 transports various organic anions, such as 17β-estradiol-17β-D-glucuronide and leukotriene $C_4$ (32), as do ABCC1, ABCC2, and ABCC3. It has also been shown that ABCC10-transfected HEK293 cells were resistant to docetaxel (33). Interestingly, this drug resistance profile of ABCC10 was greatly different from those reported for ABCC1, ABCC2, ABCC3, and ABCC6. At the present time, the actual physiological substrate of ABCC10 is unknown. Our study has addressed the expression profile of ABCC10 in various tissues and cell types to demonstrate the ubiquitous expression of this novel ABC transporter. The present study provides evidence of a new aspect that induction of the *ABCC10* gene is regulated by p53 or its signaling pathways during DNA-damage-related apoptosis. Kao et al has reported that basal expression of human *ABCC10* gene is regulated by two regions ( −1,780 to −1,287 bp and −611 to −208 bp) in the 5'-flanking region (34). It has not yet been elucidated whether these regions are involved in the up-regulation of ABCC10 gene during apoptosis. In this context, molecular mechanisms underlying the up-regulation of ABCC10 expression should be investigated in further studies.

## ACKNOWLEDGEMENTS

This study was supported by a research grant entitled "Toxicoproteomics: Expression of ABC transporter genes and drug-drug interactions" (H14-Toxico-002) from the Japanese Ministry of Health and Welfare, a Grant-in-Aid for Creative Scientific Research (No. 13NP0401) and a research grant (No. 14370754) from the Japan Society for the Promotion of Science.

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*Int. J. Cancer:* **110**, 921–927 (2004)


Publication of the International Union Against Cancer

# NOVEL CAMPTOTHECIN ANALOGUES THAT CIRCUMVENT ABCG2-ASSOCIATED DRUG RESISTANCE IN HUMAN TUMOR CELLS

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**Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin; CPT-11) is a widely used potent antitumor drug that inhibits mammalian DNA topoisomerase I (Topo I); however, overexpression of ABCG2 (BCRP/MXR/ABCP) can confer cancer cell resistance to SN-38, the active form of CPT-11. We have recently demonstrated that plasma membrane vesicles prepared from ABCG2-overexpressing PC-6/SN2-5H cells transported SN-38 and its glucuronide conjugate in an ATP-dependent manner (Nakatomi *et al.*, Biochem Biophys Res Commun 2001;288:827–32). In the present study, we have characterized a total of 14 new camptothecin (CPT) analogues with respect to both the inhibition of Topo I and the substrate specificity of ABCG2. All of the tested CPT analogues, which have different substitutions at positions 10 and 11, strongly inhibited the Topo I activity in a cell-free system, as did SN-38. Their antitumor activities in the SN-38-resistant PC-6/SN2-5H2 cell line greatly varied, however, being correlated with intracellular accumulation levels. We have examined ATP-dependent transport of those CPT analogues by using plasma membrane vesicles prepared from both PC-6/SN2-5H2 cells and ABCG2-transfected HEK-293 cells. Based on the substrate specificity of ABCG2 thus evaluated, it is strongly suggested that CPT analogues with high polarity are good substrates for ABCG2 and are therefore effectively extruded from cancer cells. In this context, to circumvent ABCG2-associated drug resistance, low-polarity CPT analogues are considered to be potent lead compounds. The present study provides a practical approach to discover new CPT-based drugs for the chemotherapy of drug-resistant human cancer.**


**Key words:** *SN-38; CPT analogue; ABCG2; SAR; DNA topoisomerase I inhibitor*

Camptothecin (CPT) is an antitumor alkaloid that was originally isolated from *Camptotheca acuminata*, a tree native to southern China.[1] CPT has been demonstrated to inhibit mammalian DNA topoisomerase I (Topo I), the nuclear enzyme that changes the topologic state of duplex DNA by single-strand breakage and resealing. Stabilization of the covalent Topo I-DNA complex (so-called cleavable complex) by CPT is a critical step in its antitumor action where Topo I-mediated DNA breaks are induced via prevention of DNA relegation.[2] Clinical and preclinical studies, however, revealed reversible bone marrow depression and hemorrhagic cystitis as the major dose-limiting toxicities of CPT. Thus, efforts have been directed at finding new CPT analogues with higher antitumor activity and less toxicity.

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin; CPT-11) has been synthesized and developed as a new water-soluble analogue of CPT with wide-spectrum antitumor activity against many types of human tumor cells.[3,4] CPT-11 is a prodrug, the antitumor activity of which is exerted by 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of CPT-11.[5] Despite enormous expense and efforts spent on the development of cancer chemotherapies, acquired and intrinsic drug resistance in tumors is the major obstacle to long-term sustained patient response to chemotherapy. Hitherto several mechanisms for the resistance to SN-38 and its analogues have been proposed, *e.g.*, mutations or decreased expression 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 protein, decreased activity of carboxylesterase that catalyzes the biosynthesis of SN-38 from CPT-11 in the plasma and liver and overexpression of drug export pumps.[6–11]

It has been documented that several ATP-binding cassette (ABC) transporters, such as P-glycoprotein (ABCB1/MDR1/P-gp) and multidrug resistance-associated protein 1 (ABCC1/MRP1), can cause drug resistance in tumor cells by actively extruding antitumor drugs.[12,13] Recently, a novel ABC transporter, ABCG2, also known as breast cancer-resistant protein (BCRP/MXR1/ABCP), has been discovered in drug-resistant cell lines selected for by mitoxantrone or Topo I inhibitors.[14,15] Overexpression of ABCG2 has been shown to confer resistance to doxorubicin, mitoxantrone and various CPT analogues.[14,16,17] In a recent study with plasma membrane vesicles prepared from ABCG2-overexpressing SN-38-selected human small cell lung carcinoma cells, we found that ABCG2 transported SN-38 and its glucuronide metabolite in an ATP-dependent manner.[18] Thus, it is highly likely that ABCG2 actively extrudes SN-38 from tumor cells and thereby confers drug resistance.

To circumvent ABCG2-associated drug resistance, in the present study we have synthesized a total of 14 different analogues of CPT and examined the substrate specificity of ABCG2 toward those analogues. Our experimental data strongly suggest that the

*Abbreviations:* ABC, ATP-binding cassette; CPT, camptothecin; CPT-11, irinotecan; HPLC, high-performance liquid chromatography; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; MTT, 3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; SAR, structure-activity relationship; SN-38, 7-ethyl-10-hydroxycamptothecin; SNP, single nucleotide polymorphism; Topo I, DNA topoisomerase I; UGT, uridine diphosphate glucuronosyltransferase.

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Received 16 October 2003; Revised 31 December 2003, 21 January 2004; Accepted 27 January 2004

DOI 10.1002/ijc.20216
Published online 24 March 2004 in Wiley InterScience (www.interscience.wiley.com).

intracellular accumulation of those analogues in the SN-38-resistant cell line is closely related to the substrate specificity of ABCG2, which is dependent on the polarity of the drug molecules here tested.

## MATERIAL AND METHODS

### *Camptothecin analogues and chemicals*

SN-38 and CPT analogues (Fig. 1) were provided by Yakult Central Institute (Kunitachi, Tokyo, Japan). Stock solutions were prepared by dissolving those compounds in dimethylsulfoxide (DMSO) at a concentration of 10 mM and stored at −30°C in the dark until used. The other chemicals used in this study were of analytical grade.

### *Cell lines*

The human small cell lung carcinoma PC-6 cells, PC-6/SN2-5H cells, the parental human T-cell-derived acute lymphoblastic leukemia cell line RPMI-8402 and the camptothecin-resistant derivative cell line CPT-K5 were maintained in RPMI-1640 medium containing 100 μg/ml kanamicin and 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere with 5% $CO_2$. The SN-38-resistant PC-6/SN2-5H2 cell line was established by continuous exposure of PC-6/SN2-5H cells to SN-38, where the concentration of SN-38 was raised from 2.5 to 25 nM in a stepwise manner. The PC-6/SN2-5H2 cell line, thus established, was then maintained in the RPMI-1640 medium supplemented with 100 μg/ml kanamicin, 10% FCS and 25 nM SN-38. It was confirmed by RT-PCR that the intrinsic expression of *ABCB1/MDR1*, *ABCC1/MRP1*, *ABCC2/MRP2* and *ABCC3/MRP3* genes was minimal in PC-6/SN2-5H cells.[19]

### *Cloning of human ABCG2 cDNA and expression in HEK293 cells*

Total RNA was extracted from PC-6/SN2-5H2 human lung carcinoma cells using the ISOGEN RNA extraction kit (Nippon Gene, Tokyo, Japan). Human ABCG2 cDNA was cloned from the RNA preparation by RT-PCR using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) and the following specific primers: sense 5−-CTCTCCAGATGTCTTCCAGT-3− and antisense 5−-ACAGTGTGATGGCAAGGGAAC-3−, where the primers were designed based on the ABCG2 cDNA sequence registered in the Genbank (accession number AF098951). The PCR reaction consisted of 30 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 2 min. The resulting PCR product was inserted into the pCR2.1 TOPO vector (Invitrogen) and its sequence was analyzed by automated DNA sequencing (TOYOBO Gene Analysis, Tokyo, Japan). Based on the cDNA sequence, the amino acid at position 141 in the cloned ABCG2 protein was substituted from Gln to Lys, as compared with the wild type of ABCG2.

| CPT analogues. | Structure X | Y |
|---|---|---|
| ○ SN-22 | H | H |
| ● SN-38 | OH | H |
| ⊗ SN-343 | Me | H |
| ⊕ SN-348 | Br | H |
| ☆ SN-349 | Cl | H |
| ★ SN-351 | H | Br |
| ◇ SN-352 | H | Cl |
| ◆ SN-353 | H | F |
| ▽ SN-355 | H | OH |
| ▼ SN-364 | Cl | Cl |
| △ SN-392 | $NH_2$ | H |
| ▲ SN-397 | OMe | F |
| □ SN-398 | OH | F |
| ■ SN-443 | Me | F |
| + SN-444 | F | F |

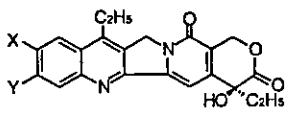


FIGURE 1 – Molecular structures of newly synthesized and tested CPT analogues.

The [Lys141]ABCG2 cDNA, thus obtained, was removed from the pCR2.1 TOPO vector by *EcoR*I digestion. After the treatment with alkaline phosphatase, ABCG2 cDNA was ligated to the *EcoR*I site of the pcDNA3.1 expression vector (Invitrogen) using the Rapid DNA ligation kit (Roche Diagnosis, Indianapolis, IN).

HEK293 cells were then transfected with the expression vector by the conventional calcium phosphate method and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μg/ml kanamycin sulfate and 10% FCS in the presence of 100 μg/ml G418 at 37°C in a humidified atmosphere with 5% $CO_2$.

### *Isolation of DNA topoisomerase I and detection of cleavable complex*

Topo I was purified from PC-6 and PC-6/SN2-5H2 cells according to Ishii *et al.*[20] Formation of the covalent Topo I-DNA complex (cleavable complex) by CPT analogues was measured according to Gagne *et al.*[8] Briefly, purified Topo I (50 units) was incubated with a CPT analogue in the reaction mixture (total volume of 50 μl) containing 0.5 μg pBluescript II plasmid DNA as the substrate, 50 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 30 μg/ml BSA and 10% glycerol. After incubation at 37°C for 15 min, the reaction was stopped by mixing with an equal volume of 2% SDS and then denatured proteins were digested by 1 mg/ml proteinase K. Cleaved DNA in the reaction mixture was separated by electrophoresis in a 1.2%-agarose gel containing ethidium bromide (0.25 μg/ml). The ratio of relaxed form I DNA (Form Ir DNA) *vs.* nicked circular DNA (Form II DNA) was measured with a densitometer (Electrophoresis Documentation and Analysis System 120, Eastman Kodak, Rochester, NY).

### *Cytotoxicity assays*

A growth-inhibition ($IC_{50}$) assay was performed by seeding cells at a density of 1,000 cells per well in 96-well plates containing the culture medium (100 μl/well). After 24 hr, drugs were added to the culture medium at different concentrations, and cells were further incubated with the drug in a humidified tissue culture chamber (37°C, 5% $CO_2$) for 72 hr. Surviving cells were detected by the MTT assay as described previously.[21] Briefly, 20 μl of bromo-3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium (MTT) solution (5 μg/ml) was added to the culture medium, and cells were incubated for 4 hr at 37°C. Thereafter, the culture medium was removed by centrifugation (700*g* at 4°C for 15 min), and the precipitate was dissolved in 200 μl of DMSO. The absorbance of formazan, a metabolite of MTT, in the resulting solution was photometrically measured at a test wavelength of 570 nm and at a reference wavelength of 630 nm in a microplate reader Model 550 (Bio-Rad, Hercules, CA). $IC_{50}$ values were calculated from dose-response curves (*i.e.*, cell survival *vs.* drug concentration) obtained in multireplicated experiments.

### *Measurement of intracellular accumulation of SN-38 analogues*

PC-6 and PC-6/SN2-5H2 cells ($1 \times 10^6$ cells) were incubated with 10 μM CPT analogues at 37°C for 10 min and CPT analogues accumulated in cells were determined as follows. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) by centrifugation at 12,000*g* for 2 min. Cells were then resuspended in 200 μl of $H_2O$ and subjected to ultrasonication. After centrifugation, CPT analogues in the resulting supernatant were measured by the HPLC method.[22] The HPLC system consisted of a Jasco PU-1580 pump, a Jasco FP-920 fluorescence detector (Jasco, Tokyo, Japan) and a Shimadzu C-R4A integrator (Shimadzu, Kyoto, Japan). Isocratic elution was performed using a Mightysil RP-18 (L) GP column (5 μm, 4.6 × 150 mm ID; Kanto Chemical, Tokyo) with a guard column (5 μm, 4.6 × 5 mm ID). The excitation and emission wavelengths were 380 and 550 nm, respectively, for SN-38, SN-398 and SN-392. The other analogues were performed emission setting at 440 nm. The mobile phase consisted of 1 mM 1-heptanesulfonic acid sodium salt in 50 mM

phosphate buffer (pH 6.0)-acetonitrile-tetrahydrofuran (THF). For separation of SN-38, SN-364 and other analogues, the ratio of mobile phases were 80:20:2 (v/v/v), 60:40:2 (v/v/v) and 70:30:2 (v/v/v), respectively. The flow-rate was 1.0 ml/min and all separations were carried out at room temperature (23–25°C).

*Preparation of plasma membrane vesicles*

Preparation of plasma membrane was performed as described previously.[18] Cells ($1 \times 10^9$ cells) were harvested by centrifugation at 8,500*g* for 10 min at 4°C and washed 3 times in PBS. The pellet was suspended in STC solution (250 mM sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$) and cells were lysed by nitrogen cavitation at 4°C under a nitrogen pressure of 900 psi for 15 min. After adding a concentrated EDTA solution to the cell lysate at a final concentration of 1 mM, the cell lysate was diluted with 7 volumes of STC solution containing 1 mM EDTA. The resulting cell lysate was overlaid on the top of a 3-step sucrose gradient consisting of 4 ml of 31%, 2.5 ml of 34% and 2.5 ml of 38% sucrose buffered with TCE (10 mM Tris-HCl, pH 7.4, 0.2 mM $CaCl_2$, 1 mM EDTA) and centrifuged at 105,000*g* for 60 min at 4°C. The membrane fraction at the interface between the 31% sucrose and the top layers was collected and washed in TCE solution by centrifugation at 100,000*g* for 60 min. The membrane was then resuspended in a small volume (50 μl) of sucrose solution (250 mM) containing 10 mM HEPES/Tris (pH 7.4), 10 mM $MgCl_2$ and 0.2 mM $CaCl_2$. The membranes were stocked at −80°C until used.

*Vesicle transport*

Transport assays were performed as described previously.[18] A suspension of plasma membrane vesicles (50 μg of protein) was incubated with 2.5 μM CPT analogues for 5 min at 37°C, after which the uptake of CPT analogues was determined by using HPLC.[22] Briefly, the transport buffer comprised CPT analogues, 250 mM sucrose, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 10 mM HEPES/Tris (pH 7.4), 10 mM creatine phosphate and 100 μg/ml creatine kinase in the presence or absence of 5 mM ATP. After preincubation for 2 min at 37°C, the vesicle suspension (50 μg protein) was incubated with the transport buffer.

The transport reaction was stopped by the addition of 800 μl of ice-cold stop buffer containing 250 mM sucrose, 100 mM NaCl, 0.2 mM $CaCl_2$ and 10 mM HEPES/Tris (pH 7.4). The stopped reaction mixture was precipitated by centrifugation at 16,000*g* for 30 min at 4°C and then washed 3 times with 1.5 ml of stop buffer. The uptake of substrate into the membrane vesicles was determined by the HPLC method.[22] The uptake in the absence of ATP is deducted from that in the presence of ATP, and an amount greater than that in parental cells from the ABCG2-overexpressing cell line is referred to as ATP-dependent uptake. The correlation coefficient is determined by fitting the data to the linear regression line. To determine the kinetic parameters, the transport study was carried out at various substrate concentrations. Kinetic parameters, such as the Michaelis-Menten constant (Km) and maximum velocity (Vmax), were calculated from Lineweaver-Burk plots.

*Immunoblotting*

Immunoblotting was performed according to Blattler *et al.*[23] and Laemmli *et al.*[24] Membrane fractions from PC-6, PC-6/SN2-5H2 and HEK293/ABCG2-141Lys cells (5 μg of protein) were loaded onto a 7.5% SDS-PAGE gel. Following transfer to PVDF membranes, immunoblotting was performed using BXP-21 antibody (1:250; Signet Laboratories, Dedham, MA) and horseradish peroxidase-conjugated antimouse IgG (1:20,000; Kirkegaard and Perry Laboratories, Gaithersburg, MD).

*Data analysis*

Spearman correlation coefficients were used to evaluate the correlation among drug resistance ratio and polarity of CPT analogues.

## RESULTS

*Inhibition of Topo I by SN-38 and CPT analogues in a cell-free system*

Before testing the effect of new CPT analogues on the activity of Topo I, we first purified the enzyme from both PC-6 and PC-6/SN2-5H2 cell lines. Figure 2(*a*) shows the SDS-PAGE photograph of the purified Topo I proteins. The molecular mass of the proteins was estimated to be 100 kDa, which is consistent with the hitherto reported molecular size.[6] Based on these SDS-PAGE results, it was concluded that contamination with other proteins was negligible in those Topo I preparations.

To test the inhibition of the Topo I activity by SN-38 as the positive control, we incubated the purified Topo I protein with pBluescript II plasmid DNA in the presence of SN-38 at various concentrations of 0.2–25 μM. As shown in Figure 2(*b*), the formation of a cleavable complex (indicated as "Form II DNA" in Fig. 2*b*) was clearly detected by agarose electrophoresis after treatment with SDS and proteinase K. The amount of the cleavable complex correlated well with the SN-38 concentration. These

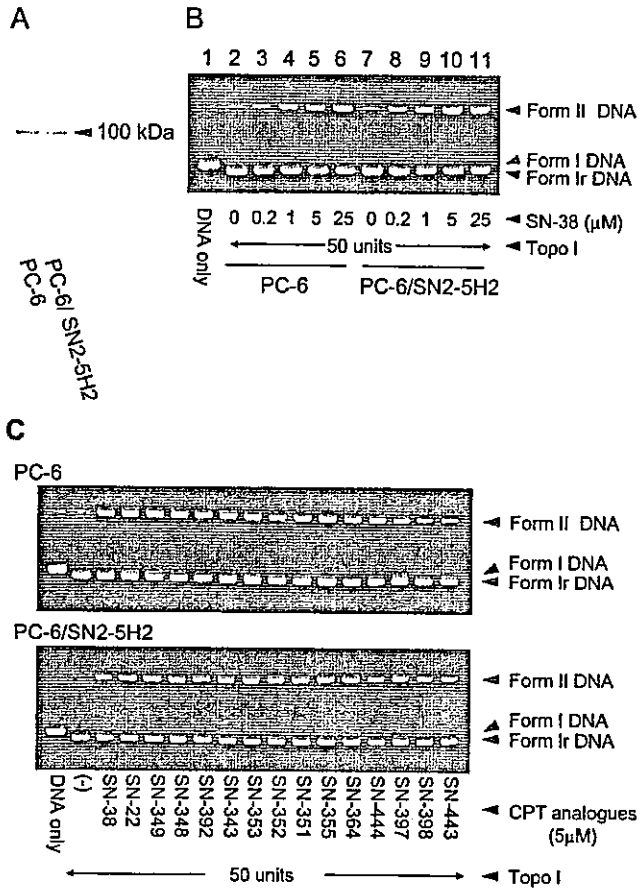


FIGURE 2 – Effect of SN-38 and SN-38 analogues on the activity of DNA Topo I purified from PC-6 (parental) and PC-6/SN2-5H2 (SN-38 resistant) cells. (*a*) SDS-PAGE showing DNA Topo I purified from PC-6 and PC-6/SN2-5H2 cells. (*b*) Effect of SN-38 on the cleavable complex (Form II DNA) formation from plasmid DNA by DNA Topo I (50 units) from PC-6 (lanes 2–6) and PC-6/SN2-5H2 (lanes 7–11) cells. Cleavable complex formation was measured in the absence (lanes 2 and 7) and presence of SN-38 at concentrations of 0–25 μM as indicated. (*c*) Effect of CPT analogues on cleavable complex formation. Cleavable complex formation was measured in the absence (−) and presence of SN-38 and CPT analogues at a concentration of 5 μM. Form I DNA, supercoiled pBluescript II plasmid DNA as the substrate; Form Ir DNA, relaxed form DNA (intrinsic DNA Topo I product).

TABLE I – EFFECT OF SN-38 AND CPT ANALOGUES ON WILD AND MUTATED TYPES OF TOPO I

| CPT analogues | $span 2-36iC$_{50}$ value (nM)[1] | |
|---|---|---|
| | RPMI-8402 | CPT-K5 |
| SN-22 | 5.48 ± 0.31 | >2,500 |
| SN-38 | 13.4 ± 2.32 | >2,500 |
| SN-343 | 3.08 ± 0.16 | >2,500 |
| SN-348 | 2.2 ± 0.23 | >2,500 |
| SN-349 | 3.17 ± 0.56 | >2,500 |
| SN-351 | 4.69 ± 2.25 | >2,500 |
| SN-352 | 4.98 ± 0.97 | >2,500 |
| SN-353 | 1.53 ± 0.35 | >2,500 |
| SN-355 | 15.5 ± 8.53 | >2,500 |
| SN-364 | 3.82 ± 2.17 | >2,500 |
| SN-392 | 3.30 ± 0.49 | >2,500 |
| SN-397 | 1.23 ± 0.02 | >1,000 |
| SN-398 | 21.8 ± 11.3 | >2,500 |
| SN-443 | 2.06 ± 0.09 | >2,500 |
| SN-444 | 1.63 ± 0.74 | >2,500 |

Drug sensitivity was determined by MTT assay after a 72-hr drug exposure. [1]$IC_{50}$ value: drug concentration representing a 50% reduction of cell growth.

TABLE II – DRUG RESISTANCE PROFILE OF PC-6 (SN-38-SENSITIVE) AND PC-6/SN2-5H2 (SN-38-RESISTANT) CELLS TO CPT ANALOGUES

| CPT analogues | $IC_{50}$ value (nM)[1] | | Drug resistance ratio[2] |
|---|---|---|---|
| | PC-6 | PC-6/SN2-5H2 | |
| SN-22 | 0.85 ± 0.11 | 2.98 ± 0.63 | 3.5 |
| SN-38 | 1.00 ± 0.10 | 262 ± 5.70 | 262 |
| SN-343 | 0.58 ± 0.08 | 1.39 ± 0.09 | 2.4 |
| SN-348 | 0.32 ± 0.08 | 0.93 ± 0.06 | 2.9 |
| SN-349 | 0.41 ± 0.01 | 2.13 ± 0.43 | 5.2 |
| SN-351 | 0.45 ± 0.10 | 2.88 ± 0.04 | 6.4 |
| SN-352 | 0.69 ± 0.08 | 1.79 ± 0.42 | 2.6 |
| SN-353 | 0.28 ± 0.03 | 1.01 ± 0.03 | 3.6 |
| SN-355 | 1.04 ± 0.25 | 109 ± 1.50 | 105 |
| SN-364 | 0.79 ± 0.06 | 1.59 ± 0.22 | 2.0 |
| SN-392 | 0.44 ± 0.06 | 24.0 ± 2.98 | 55 |
| SN-397 | 0.09 ± 0.01 | 1.14 ± 0.07 | 13 |
| SN-398 | 0.41 ± 0.11 | 87.4 ± 8.80 | 213 |
| SN-443 | 0.85 ± 0.20 | 1.52 ± 0.04 | 1.8 |
| SN-444 | 0.46 ± 0.08 | 2.19 ± 0.34 | 4.8 |

Drug sensitivity was determined by MTT assay after a 72-hr drug exposure. [1]$IC_{50}$ value: drug concentration representing a 50% reduction of cell growth.– [2]Drug resistance ratio: calculated as $IC_{50}$ for PC-6/SN2-5H2/$IC_{50}$ for PC-6.

results strongly suggest that SN-38 inhibited Topo I purified from both PC-6 and PC-6/SN2-5H2 cell lines in a dose-dependent manner.

Figure 2(*c*) demonstrates the effect of SN-38 and CPT analogues on the formation of the cleavable complex. At a concentration of 5 μM, the amount of the cleavable complex was estimated to be about 40% of the total pBluescript II plasmid DNA for all of the CPT analogues tested. In addition, there was no significant difference in the cleavable complex formation between the Topo I preparations from PC-6 and PC-6/SN-2-5H2 (Fig. 2*c*). Based on these results, it is concluded that those SN-38 analogues inhibited Topo I in the cell-free system with almost equal potency.

*Effect of CPT analogues on wild and mutated types of topo I*

To examine the inhibitory effect of CPT analogues on Topo I at the cellular level, we used 2 different cell lines, namely, RPMI-8402 and CPT-K5. The former is a human T-cell-derived acute lymphoblastic leukemia cell line bearing the wild type of Topo I, whereas the latter is the camptothecin-resistant derivative cell line derived from RPMI-8402 and carrying a mutant form of Topo I.[6,25] Those cell lines were incubated in the presence of SN-38 and the CPT analogues for 72 hr, and their cell growth was measured by the MTT assay. As shown in Table I, the RPMI-8402 cell line was sensitive to the CPT analogues in the low concentration range; $IC_{50}$ values were estimated to be from 1.23 to 21.8 nM. In contrast, the CPT-K5 cell line was insensitive to SN-38 as well as to all the analogues tested at even much higher concentrations (> 2,500 nM). It is documented that the Gly-533 mutation of Topo I confers the CPT-K5 cell line camptothecin resistance by inhibition of CPT binding.[26] Thus, our results strongly suggest that those CPT analogues specifically inhibit the wild type of Topo I, as does SN-38.

*Cellular accumulation of CPT analogues and drug resistance profile*

In our previous study, we demonstrated that ABCG2 expressed in PC-6/SN2-5H cells transported SN-38 in an ATP-dependent manner, and it has suggested that overexpression of such an export pump is one of the resistance factors of utmost concern with respect to SN-38 resistance.[18] In the present study, we have screened CPT analogues by using both PC-6/SN2-5H2 (SN-38-resistant) and PC-6 (SN-38-sensitive) cell lines to gain insight into the relationship between the drug resistance profile and cellular accumulation. Table II lists the $IC_{50}$ values and drug resistance ratios for CPT analogues as determined by the MTT assay method. In the positive control, PC-6/SN2-5H2 was approximately 260-fold more resistant to SN-38 than was PC-6 (drug resistance ratio: 262). As shown in Table II, the $IC_{50}$ values of most CPT analogues (except for SN-355) were lower than that of SN-38 in PC-6 cells. In particular, SN-397 was the most potent ($IC_{50}$ = 0.09 nM) among the CPT analogues tested. On the other hand, in the SN-38-resitant PC-6/SN2-5H2 cell line, 3 analogues, *i.e.*, SN-392, SN-355 and SN-398, exhibited relatively high $IC_{50}$ values. Indeed, the drug resistance ratios of those 3 analogues were in the range of 55–213. It is noteworthy that the other analogues exhibited low drug resistance ratios (Table II), suggesting that those analogues could circumvent SN-38 resistance.

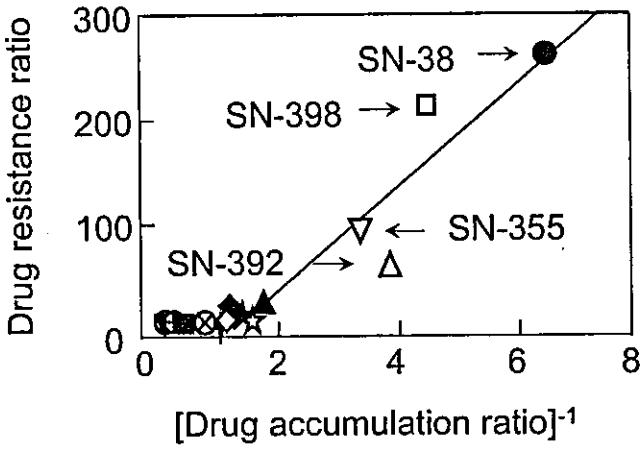


FIGURE 3 – Correlation between drug resistance ratio and drug accumulation ratio. Drug accumulation in PC-6 and PC-6/SN2-5H2 was measured by HPLC after incubation with 10 mM CPT analogues for 10 min at 37°C. See Figure 1 for the symbols of CPT analogues. Drug resistance ratio is calculated from the ratio of $IC_{50}$ (PC-6/SN2-5H2)/$IC_{50}$ (PC-6). Drug accumulation ratio is drug accumulation (PC-6/SN2-5H2)/drug accumulation (PC-6).

To confirm whether such drug resistance profiles are related to the intracellular accumulation of CPT analogues, we measured the amounts of CPT analogues accumulated in both PC-6 and PC-6/SN2-5H2 cells by our recently established HPLC method.[22] Figure 3 demonstrates a strong correlation (r = 0.95) between drug resistances ratios and inverse values of drug accumulation ratios. The drug accumulation ratio is defined as a relative value calculated from the drug accumulation level in PC-6/SN2-5H2 cells divided by that in PC-6 cells. Likewise, the drug resistance ratio is

calculated from the $IC_{50}$ value of PC-6/SN2-5H2 cells divided by that of PC-6 cells. As already shown in Table II, SN-38, SN-392, SN-355 and SN-398 exhibited high drug resistance ratios. In this regard, Figure 3 clearly demonstrates that such high levels of drug resistance in PC-6/SN2-5H2 cells are closely associated with reduced levels of intracellular accumulation of those analogues. On the other hand, it is important to note that the cellular accumulation of the other CPT analogues was not very much reduced in PC-6/SN2-5H2 cells. This may explain the observation that those analogues effectively inhibited the growth of SN-38-resistant PC-6/SN2-5H2 cells (Table II).

*ATP-dependent transport of CPT analogues in plasma membrane vesicles*

To elucidate our hypothesis that the reduced intracellular drug accumulation of SN-392, SN-355 and SN-398 is due to active extrusion mediated by ABCG2 expressed in PC-6/SN2-5H2 cells, we measured the ATP-dependent transport of those CPT analogues by using plasma membrane vesicles prepared from PC-6 and PC-6/SN2-5H2 cells as well as from [Lys141]ABCG2-transfected HEK293 cells. Figure 4 demonstrates the relationship between the drug resistance ratios and ATP-dependent transport of those analogues in membrane vesicles from PC-6/SN2-5H2 cells. A high correlation was obtained between the drug resistance ratios and ATP-dependent transport. In addition, we analyzed the kinetic parameters for the ATP-dependent transport of those analogues by PC-6/SN2-5H2 and HEK293/ABCG2-Lys141 cells. As shown in Table III, the calculated Vmax/Km values of those analogues (indicated by V/K in the table) are intimately related to their drug resistance ratios. Based on these results, it is suggested that the drug resistance and reduced intracellular accumulation of those analogues is due to their ABCG2-mediated active export from the cell.

*Polarity of CPT analogues* vs. *drug resistance ratio*

During the HPLC measurement of CPT analogues accumulated in PC-6 and PC-6/SN2-5H2 cells, we have noticed that both drug resistance profile and cellular accumulation were associated with the polarity of those analogues. Since we used a reverse-phase column for the HPLC measurement, CPT analogues such as SN-38, SN-355, SN-392 and SN-398 were eluted with a short retention time, and those analogues exhibited high drug resistance ratios (Table II) and reduced levels of cellular accumulation (Fig. 3). Figure 5 shows the relationship between the drug resistance ratio and the HPLC retention time for all CPT analogues tested in this study.

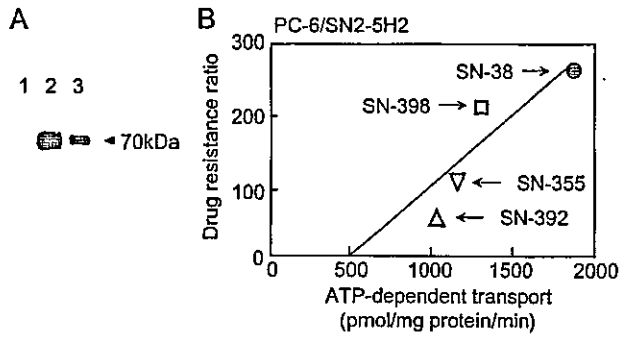


FIGURE 4 – Correlation between drug resistance ratio and ATP-dependent transport. (*a*) Immunoblot detection of ABCG2 in plasma membrane preparations. Lane 1, PC-6 (SN-38-sensitive); lane 2, PC-6/SN2-5H2 (SN-38-resistant); lane 3, HEK293/ABCG2-Lys141 cells transfected with [Lys141]ABCG2 cDNA. Arrowhead indicates the ABCG2 protein. (*b*) Correlation between drug resistance ratio and ATP-dependent transport of PC-6/SN2-5H2. Drug resistance ratio is calculated from the ratio of $IC_{50}$ (PC-6/SN2-5H2)/$IC_{50}$ (PC-6). ATP-dependent transport for PC-6 from that for PC-6/SN2-5H2.

The Spearman correlation coefficient was calculated to evaluate correlation between the polarity of those analogues and the drug resistance ratio. The result (Spearman r = 0.65) suggests that the drug resistance ratio is positively correlated with the polarity of the analogues tested in this study, namely, the polarity is considered to be an important factor for both drug resistance and the cellular accumulation of those analogues in SN-38-resistant cells, *e.g.*, PC-6/SN2-5H2 cells.

## DISCUSSION

*Characterization of SN-38 analogues in a cell-free system*

CPT-11 is one of the prominent antineoplastic drugs widely used in clinical practice today. CPT-11 *per se* is a prodrug and undergoes carboxylesterase-mediated hydrolysis to form SN-38, a potent Topo I inhibitor. It has been reported that the lactone E ring of SN-38 is an important pharmacophor for the inhibition of Topo I. Hydrolysis of the lactone E ring results in remarkable reduction of antitumor activity of SN-38. In contrast, modifications of the A or B rings do not significantly affect Topo I inhibition activity.[27] In this context, we have synthesized CPT analogues with various substitutions at positions 10 or 11 of the A ring, as demonstrated in Figure 1. All of the new CPT analogues inhibited the wild type of Topo I, as does SN-38 (Tables I and II). Their activities to form the cleavable complex were almost equal to that of SN-38 (Fig. 2*c*). Therefore, it is concluded that all of the CPT analogues are as potent as SN-38 in the cell-free system.

*CPT analogues that circumvent ABCG2-associated drug resistance*

PC-6/SN2-5H2 cells were 260-fold more resistant to SN-38 than were PC-6 cells. This drug resistance was conferred by the reduced accumulation of SN-38 in the cell. In the present study, PC-6/SN2-5H2 cells were found to be resistant to SN-355, SN-392 and SN-398 as well (Table II). Reduced intracellular accumulation of those CPT analogues was closely related to the drug resistance profile (Fig. 3). On the other hand, it is important to know that the other analogues exhibited effective antitumor activity against PC-6/SN2-5H2 cells. Indeed, analogues could circumvent the drug resistance. Therefore, we tried to gain insight into molecular aspects that would differentiate between the drug resistance profiles of the CPT analogues.

In our previous study, we provided evidence that expression of ABCG2 is involved in the drug resistance to SN-38. ABCG2 is a member of the G family of ABC transporters and transports SN-38 out of the cell in an ATP-dependent manner.[18] The present study demonstrates that membrane vesicles from PC-6/SN2-5H2 transported SN-38, SN-355, SN-392 and SN-398 in an ATP-dependent manner (Fig. 4). ATP-dependent transport of SN-38 was inhibited by novobiocin and quercetin.[19,28] As demonstrated in Table III, ABCG2 cloned from PC-6/SN2-5H2 cells also transported SN-38, SN-355, SN-392 and SN-398 in an ATP-dependent manner. ABCG2-transfected HEK293 cells were resistant to SN-38, SN-355, SN-392 and SN-398, but not to the other analogues tested (data not shown). Based on these findings, it is speculated that SN-38, SN-355, SN-392 and SN-398 have common properties in their molecular structure. In fact, SN-38 and SN-398 have a hydroxyl group at position 10, whereas SN-355 has one at position 11 (Fig. 1). In addition, SN-392 has an amino group at position 10 (Fig. 1). Hydroxyl and amino groups are important for the formation of hydrogen bonds. Interestingly, the other CPT analogues do not have such groups at positions 10 or 11 (Fig. 1). It is likely that hydrogen bond formation may be involved in substrate recognition and/or the transport processes of ABCG2.

Analysis of the structure-activity relationship (SAR) among CPT-based analogues is of interest to understand the substrate specificity of ABCG2 and also to design new antitumor drugs that circumvent ABCG2-associated drug resistance. In the present study, we found a good correlation between the drug resistance ratio and the polarity of CPT analogues (Fig. 5). CPT analogues