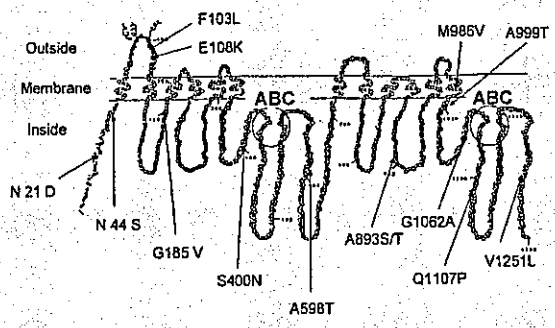

Fig. 4: Schematic representation of ABCB1 and non-synonymous polymorphism. The positions of amino acid substitutions are indicated by arrows. The molecular structure of ABCB1 is modified from Gottesman MM, Pastan I: The multidrug transporter, a double-edged sward. J. Biol. Chem. 263, 12153-12166 (1988). ABC, ATP-binding cassette.

ABCB1 gene. One of those mutations in particular, a C-to-T variant at position 3435 in the exon 26 of the *ABCB1* gene, was reportedly correlated with ABCB1 expression and function. While this SNP does not alter the amino acid sequence of ABCB1, individuals homozygous for the polymorphism expressed significantly less duodenal ABCB1 and significantly more plasma digoxin [15]. The distribution of the SNP C3435T in exon 26 in the Chinese and Malay population was found to be similar to the Caucasians whereas the Indians were different. The Asian population also differed significantly from the African and Caucasian population in the distribution of the C3435T SNP [16,17]. However, the association of the C3435T polymorphism with ABCB1 protein expression and function remains controversial. In fact, various investigators have reported that the T allele is associated with increased (or decreased) expression levels or has no clearly discernible effect. Furthermore, the reported effects of C3435T SNP on the pharamcokinetic profiles of drugs are also controversial (see 18,19 for recent reviews).

Recently, five most common coding SNPs were assessed by a vaccinia virus-based transient expression system [20]. The study on cellular accumulation of several tested substrates indicated that the substrate specificity of the protein was not substantially affected by any of the SNPs, whereas cell surface expression and function of even double mutants showed no difference from the wild-type protein. The study suggests that these SNPs result in mutant proteins with a distribution and function similar to the wild-type protein. However, kinetic parameters for those variants have not been assessed in that study.

On the other hand, the ABCB1 G2677T/A [21] contains a tri-allelic polymorphism (with G at nucleotide 2677 found in the wild-type sequence, and with A or T at that position being the two possible variants), which results in an amino acid change (A893S/T) in exon 21. Previous work has shown that the Ser893 substitution is associated with an altered drug resistance pattern in AdrR MCF-7 cells as well as enhanced efflux transporting ability in stably transfected NIH3T3 GP + E86 cells [22].

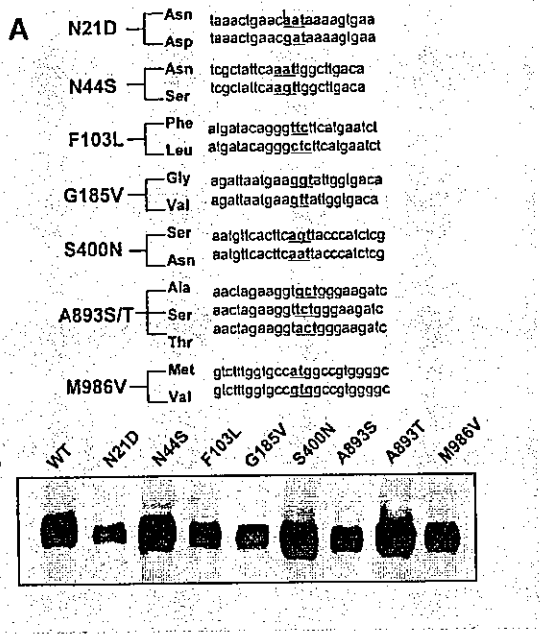

Fig. 5: Expression and functional analysis of ABCB1 variants. **A.** PCR primers used for site-directed mutagenesis. The sequences are compared with those of the wild type ABCB1. The codons corresponding to amino acid substitutions are indicated by underlines. **B.** Western blotting analysis for ABCB1 variants expressed in the plasma membrane fraction of Sf9 cells.

Acquired mutation of ABCB1 in cancer cells

Cells selected *in vitro* against a lipophilic cytotoxic compound usually develop cross-resistance to other drugs. Some multidrug-resistant cell lines are significantly more resistant to the drug used in their selection than to the other drugs. A single amino acid substitution, Gly185Val, in the human ABCB1 protein was found to cause an altered pattern of drug resistance in cell lines transfected with the ABCB1 cDNA carrying this mutation [23]. It is suggested that the amino acid at position 185 is involved in colchicines and verapamil but not in vinblastine binding/transport. In addition, several recombinant variants have been generated either by *in vivo* drug selection or by site-directed mutagenesis techniques, which show altered substrate specificity or impaired function of a properly assembled protein.

Functional analysis of ABCB1 polymorphism

Fig. 4 depicts hitherto identified nonsynonymous polymorphisms in the ABCB1 protein. Quantitative studies are required to precisely evaluate functional changes associated with genetic polymorphisms of ABCB1. For this purpose, the cDNA of ABCB1 was cloned from the human liver cDNA library, and several variant forms (i.e., N21D, N44S, F103L, G185V, S400N, A893S, A893T, M986V) were prepared by site-directed mutagenesis (see **Fig. 5A** for primers). These variants and the wild type of ABCB1 were then expressed it in Sf9 cells using the pFASTBAC1 vector and recombinant baculoviruses. ABCB1 variant proteins expressed in Sf9

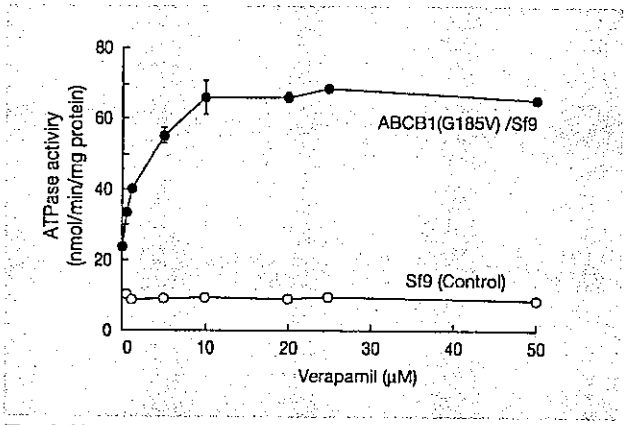

Fig. 6: Verapamil-enhanced ATPase activity in the plasma membrane fraction of Sf9 cells. Closed circles, ABCB1 (G185V)-expressing Sf9 cells; open circles, control Sf9 cells.

cell membranes were detected by the western blot method using a specific monoclonal antibody (**Fig. 5B**).

Using membranes prepared from Sf9 cells expressing ABCB1 variants, ATPase activity of this ABC transporter was measured in the presence of various compounds. For this purpose, we have recently developed a high throughput screening system using 96-well plates, where the ATPase activity in the isolated Sf9 cell membranes is determined by measuring inorganic phosphate liberation [24]. As demonstrated in **Fig. 6**, verapamil, one of the typical substrates of ABCB1, enhanced ATPase activity. **Table 2** summarizes kinetic parameters observed for the variant forms as well as the wild type of ABCB1. The variant forms (i.e., N21D, N44S, F103L, G185V, S400N, A893S, A893T, M986V) exhibited the verapamil-enhanced ATPase activity, as did the wild type of ABCB1. Km values for verapamil were slightly different among those variants. The Vmax values of the variants were normalized to that of the wild type by referring to the intensity of each variant protein on the western blotting (**Fig. 5B**). The variant G185V (acquired mutation) was found to have the highest Vmax value, which was followed by N21D (**Table 2**). Thus, it is critically important to quantitatively analyze the functional difference among such variants in evaluating naturally occurring nonsynonymous polymorphisms. In addition, 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. Kinetic parameters of those variants observed with different substrates will be reported elsewhere.

ABCB1 gene expression and its clinical impact

There are many factors that can affect not only the function but also the expression levels of drug transporters. Numerous environmental factors affecting the phenotypical activity of drug transporters must be considered, which may include exogenous chemicals, food constituents, herbal preparations, and/or therapeutic drug use that may induce or inhibit the function or expression of drug transporters,

Variant	Km	Vmax
	(μM)	(nmol/min/mg protein)
Wild type	2.190±0.150	13.14±1.95
N21D	0.502±0.126	45.26±11.33
N44S	0.580±0.148	31.03±4.65
F103L	1.100±0.078	36.34±8.33
G185V	0.831±0.102	56.76±6.76
S400N	0.327±0.025	13.74±2.08
A893S	0.441±0.042	17.24±6.72
A893T	0.904±0.244	10.77±1.35
M986V	0.419±0.062	22.69±6.84

Table 2: Kinetic parameters of the wild type and SNP variants of ABCB1

The wild type and variants of ABCB1 were then expressed it in Sf9 cells using the pFASTBAC1 vector and recombinant baculoviruses.
Using membranes prepared from Sf9 cells expressing ABCB1 variants, ATPase activity was measured in the presence of verapamil at different concentrations. Km and Vmax values were calculated from Lineweaver-Burk plots. ABCB1 proteins expressed in Sf9 cell membranes were detected by the western blot method using the C219 monoclonal antibody. The Vmax values of the variants were normalized to that of the wild type by referring to the intensity of each variant protein on the western blotting. Data are expressed as mean values ± S.D. (n = 3).

as exemplified by the cases of ABCB1 [25,26].

Transcription of the *ABCB1* gene appears to be regulated by multiple factors. For example, the proximal promoter region has a GC-rich region, at approximately −100 to −120 bp from the transcriptional start codon that contains a site responsible for the repression of transcription. Also, basal transcription appears to involve a consensus site that binds NF-Y transcription factors at a Y-box (inverted CCAAT box) between −70 and −80 bp. In addition, a binding site for SP-1 and members of the early growth response (EGR) family of transcriptional factors is present which overlaps with the NF-Y consensus site. A 13-bp region around the initiation site involved in accurate initiation of the transcription has also been identified. ABCB1 is expressed at a high frequency in tumor cells and both c-H-Ras and mutant forms of p53 have been shown to activate the *ABCB1* promoter. On the other hand, c- and N-Myc expression is apparently inversely correlated with ABCB1 expression. In addition to such transcriptional regulations, the stability of mRNA and posttranslational regulation are also considered important in the regulation of ABCB1 expression.

Variation in the pharmacokinetic behavior of a drug among different patients is the net results of complex interactions between genetic, physiological, and environmental factors. If the function or expression level of drug transporters is altered due to genetic factors, intestinal secretion of the drug into the gut lumen may change. Such information may be valuable in predicting an increase or decrease in bioavailability or orally administered substrate drugs in individual patients. In the case of organ transplantation, it is critically important to maintain the concentration of an immunosuppressive drug at sufficient levels in the blood circulation. The success of the transplantation depends on a delicate balance between immunosuppression and rejection. For instance, tacrolimus (FK506) is a widely used immunosuppressor,

whereas large variability has been noted in its bioavailability after oral administration of this drug. Recent studies have provided evidence that intestinal expression of ABCB1 is a good probe for prediction of the interindividual variation in tacrolimus pharamcokinetics after organ transplantation and also a powerful prognostic indicator for the outcome of organ transplantation [27,28].

Future perspectives

In the near future, more information on SNPs and haplotype maps will be available for a variety of drug transporters. It will become increasingly important to determine which polymorphism has influence on the function and gene expression of drug transporters. The expression and phenotypical activity of drug transporters in patients may be strongly influenced by medications. Genetic variations in the promoter region or introns of drug transporter genes may alter the gene expression. In addition, non-genetic and/or epigenetic factors are also considered to have potential effects. Gene regulation of drug transporters as well as drug metabolizing enzymes is of great interest to understand the molecular mechanisms of drug response and toxic events. It has been documented that hydrophobic ligands and several nuclear receptors are involved in induction or down-regulation of cytochrome P-450 isoforms and ABC transporters [29,30]. While the gene regulation of drug metabolizing enzymes, such as cytochrome P-450, glucuronide transferases and glutathione transferases, have been well characterized, pharmacogenomics studies on the molecular mechanisms underlying the induction and/or down-regulation of drug transporters would significantly contribute to our understanding of individual difference in drug response.

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Review

Pharmacogenomics of Drug Transporters: A New Approach to Functional Analysis of the Genetic Polymorphisms of ABCB1 (P-Glycoprotein/MDR1)

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Received March 31, 2004

In the 21st century, emerging genomic technologies (*i.e.*, bioinformatics, functional genomics, and pharmacogenomics) are shifting the paradigm of drug discovery research and improving the strategy of medical care for patients. In order to realize the personalized medicine, it is critically important to understand molecular mechanisms underlying inter-individual differences in the drug response, namely, pharmacological effect *vs.* side effect. Evidence is now accumulating to strongly suggest that drug transporters are one of the determinant factors governing the pharmacokinetic profile of drugs. Effort has been made to identify genetic variation in drug transporter genes. In particular, genetic variations of the human *ABCB1* (*P-glycoprotein/MDR1*) gene have been most extensively studied. Hitherto more than fifty single nucleotide polymorphisms (SNPs) and insertion/deletion polymorphisms in the *ABCB1* gene have been reported. However, at the present time, information is still limited with respect to the actual effect of those genetic polymorphisms on the function of ABCB1. In this context, we have undertaken functional analyses of ABCB1 polymorphisms. To quantify the impact of genetic polymorphisms on the substrate specificity of ABCB1, we have developed a high-speed screening system and a new structure–activity relationship (SAR) analysis method. This review addresses functional aspects of the genetic polymorphism of ABCB1 and provides the standard method to evaluate the effect of polymorphisms on the function.

Key words P-glycoprotein; single nucleotide polymorphism; high throughput screening

INTRODUCTION

Pharmacogenomics, the study of influence of genetic factors on drug action, is increasingly important for predicting pharmacokinetics profiles and/or adverse reactions of drugs. Drug transporters and drug-metabolizing enzymes are recognized as significant factors, because they play pivotal roles in determining the pharmacokinetic profiles of drugs and, by extension, their overall pharmacological effects (*i.e.*, drug absorption, drug distribution, drug metabolism and elimination, drug concentration at the target site, and the number and morphology of target receptors).[1—10] The effects of drug transporters on the pharmacokinetic profile of a drug depend on their expression and functionality. Indeed, the expression of drug transporters can be modulated by endogenous and exogenous factors, including drugs, themselves. It is also now known that inherited differences among individuals may also affect drug efficacy and toxicity.[11—13] Such inherited differences include genetic polymorphisms in drug targets and drug-metabolizing enzymes, as well as in drug transporters (Fig. 1). Hitherto, pharmacogenetics, the field dealing with such inherited differences and their effect on pharmacokinetics, has significantly contributed to our understanding of genetic causes underlying differences in drug metabolism (*e.g.*, cytochrome P-450 mediated drug metabolism). In fact, recent technological advances allowing massive molecular sequencing have in turn allowed us to identify single nucleotide polymorphisms (SNPs) as one possible cause of variable drug response among individuals.[14,15] In light of such advances, it is important to carefully examine the clinical significance, if any, of polymorphisms in drug response genes, including drug transporters.

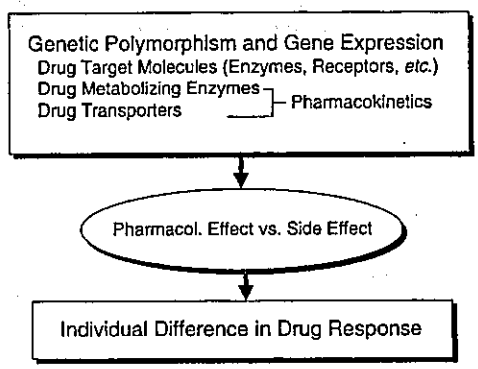

Fig. 1. Impact of the Genetic Polymorphism and/or Gene Expression of Drug Targets, Drug Metabolizing Enzymes, and Drug Transporters on the Drug Response

There are an increasing number of literatures that address genetic polymorphisms of drug transporters. At present, concerning the effects of genetic polymorphisms on pharmacokinetic profiles, the best characterized transporter is ABCB1. However, it is also true that there is still considerable discrepancy among hitherto reported results. There are many factors that can affect the function as well as the expression of drug transporters. Those factors may involve genetic mutations, SNPs, splicing, transcriptional regulation, stability of mRNA, post-translational modification, and intracellular localization. Evaluation of such factors is critically important to understand the whole picture of pharamacogenomics of drug transporters. Functional analysis of the polymorphism of drug transporters is one of such important approaches that provide clear insight into the biochemical significance of genetic polymorphisms.[16] Figure 2 summarizes the strategy of

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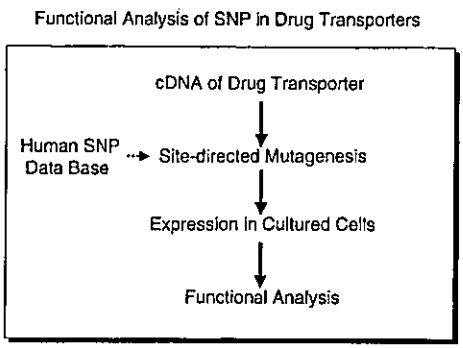

Fig. 2. Strategy for the Functional Analysis of Non-synonymous SNPs in Drug Transporters

our functional analysis. 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. The present review article provides a new approach of quantitative analysis for the genetic polymorphism of ABCB1 (P-glycoprotein or MDR1) and its functionality.

1. ABCB1 (P-GLYCOPROTEIN/MDR1)

Human ABCB1 (P-glycoprotein or MDR1) was identified because of its overexpression in cultured cancer cells associated with an acquired cross-resistance to multiple anticancer drugs.[17] While "P-glycoprotein" was initially thought to play a role in modulating cellular permeability ('P' stands for permeability) to drugs, it has later been demonstrated to be an ATP-dependent efflux pump of hydrophobic anticancer drugs including colchitine, doxorubicin, daunorubicin, vincristiene and VP16. Historically P-glycoprotein provided one of the mechanistic explanations for the multidrug resistance phenomenon. The function of ABCB1 as a mechanism of multidrug resistance has been extensively investigated.[18]

1.1. Molecular Structure Human ABCB1 and its orthologues in mammalians are single peptide chains, integral membrane proteins of an approximate length of 1280 amino acid residues. The apparent molecular weight of mature ABCB1 ranges from about 130 to 180 kDa, depending on the species and cell type in which they are expressed. ABCB1 is composed of two homologous halves each of which consist of an N-terminal, hydrophobic, membrane-associated domain (approximately 250 amino acid residues) and a C-terminal, hydrophilic nucleotide binding fold (approximately 300 amino acid residues). The plasma membrane associated domains in the two halves of ABCB1 each consist of six transmembrane domains, which are followed by an intracellular ATP-binding cassette.

In order to elucidate the transport mechanism and structure of ABCB1, mutational analysis was widely carried out using site-directed mutagenesis. A relatively large number of mutants alter the transporter's substrate specificity, in particular those in transmembrane domains 5, 6, and 12. Accordingly, photo-affinity labeling studies indicated that such domains probably are of major importance in substrate binding.

1.2. Gene Structure Humans *ABCB1* and *ABCB4* genes are adjacently located on chromosome 7q21. *ABCB1* encodes a drug transporter directly associated multidrug resistance of cancer, whereas *ABCB4* encodes the flippase translocating phopholipids. In rodents, three genes are present: *mdr1a*, *mdr1b*, and *mdr2*. In the mouse, the genes are clustered on chromosome 5 whereas they are located at chromosomal region 4q11-12 in the rat. In rodents, both Mdr1a and Mdr1b functionally correspond to ABCB1 (MDR1) in humans.

Transcription of the *ABCB1* gene appears to be regulated by multiple factors. For example, the proximal promoter region has a GC-rich region, at approximately −100 to −120 bp from the transcriptional start codon, that contains a site responsible for the repression of transcription. Also, basal transcription appears to involve a consensus site that binds NF-Y transcription factors at a Y-box (inverted CCAAT box) between −70 and −80 bp. In addition, a binding site for SP-1 and members of the early growth response (EGR) family of transcriptional factors is present which overlaps with the NF-Y consensus site. A 13-bp region around the initiation site involved in accurate initiation of the transcription has also been identified. ABCB1 is expressed at a high frequency in tumor cells and both c-H-Ras and mutant forms of p53 have been shown to activate the *ABCB1* promoter. On the other hand, c- and N-Myc expression is apparently inversely correlated with ABCB1 expression. In addition to such transcriptional regulations, the stability of mRNA and posttranslational regulation are also considered important in the regulation of ABCB1 expression.

1.3. ABCB1 in Normal Tissues It is important to know that ABCB1 is expressed not only in cancer cells but also in many normal tissues. For example, it is located in the apical domain of the enterocytes of the gastrointestinal tract (jejunum and duodenum) and limits the uptake and absorption of drugs and other substrates from the intestine into the systemic circulation by excreting substrates into the gastrointestinal tract. In addition, ABCB1 is expressed in the endothelial cells lining the small vessels of the human cortex, in which the transporter appears concentrated within the luminal cellular compartment.[19] The expression of ABCB1 on the lumenal membrane of capillary endothelial cells of the brain restricts drug distribution into the central nervous system. This function of ABCB1 appears to be very important in protecting the central nervous system from the attack of toxic compounds. Evidence for the protective role of ABCB1 in the blood-brain barrier has been demonstrated in several studies using *mdr1a* knockout mice.[20] A similar protective role to limit the distribution of potentially toxic xenobiotics into tissues was suggested for ABCB1 expressed in the placenta and the testis. ABCB1 expressed in the canalicular domain of the hepatocyte and the brush border of the proximal renal tubule plays a role in the biliary and urinary excretion of xenobiotics and endogenous compounds.

1.4. Acquired Mutation of ABCB1 in Cancer Cells Cells selected *in vitro* against a lipophilic cytotoxic compound usually develop cross resistance to other drugs. Some multidrug-resistant cell lines are significantly more resistant to the drug used in their selection than to the other drugs. A single amino acid substitution, Gly185Val, in the human ABCB1 protein was found to cause an altered pattern of drug resistance in cell lines transfected with the ABCB1 cDNA carrying this mutation.[21] It is suggested that the amino acid at position 185 is involved in colchicines and verapamil but

not in vinblastine binding/transport. In addition, several recombinant variants have been generated either by *in vivo* drug selection or by site-directed mutagenesis techniques, which show altered substrate specificity or impaired function of a properly assembled protein.[18]

1.5. Naturally Occurring SNPs of ABCB1 To date, genetic variations of the human *ABCB1* gene have been most extensively studied (ref. 22, 23 for recent reviews). Hitherto 50 SNPs and 3 insertion/deletion polymorphisms in the *ABCB1* gene have been reported.[24—27] In addition, twelve novel SNPs of ABCB1 were reported in Japanese patients with ventricular tachycardia who were administered amiodarone.[28] Several preclinical and clinical studies have provided evidence for the naturally occurring polymorphisms in ABCB1 and their effects on drug absorption, distribution and elimination.[29—62] Hoffmeyer *et al.* reported multiple polymorphisms in the *ABCB1* gene.[38] One of those mutations in particular, a C-to-T variant at position 3435 in the exon 26 of the *ABCB1* gene, was reportedly correlated with ABCB1 expression and function. While this SNP does not alter the amino acid sequence of ABCB1, individuals homozygous for the polymorphism expressed significantly less duodenal ABCB1 and significantly more plasma digoxin.[38] The distribution of the 3435C>T SNP in exon 26 in the Chinese and Malay population was found to be similar to the Caucasians whereas the Indians were different. The Asian population also differed significantly from the African and Caucasian population in the distribution of the 3435C>T SNP.[39,40] However, the association of the 3435C>T polymorphism with ABCB1 protein expression and function remains controversial. In fact, various investigators have reported that the T allele is associated with increased (or decreased) expression levels or has no clearly discernible effect.[41—48] Furthermore, the reported effects of the 3435C>T SNP on the pharmacokinetic profiles of drugs are also controversial.[49—62]

Recently, five most common coding SNPs were assessed by a vaccinia virus-based transient expression system.[63] The study on cellular accumulation of several tested substrates indicated that the substrate specificity of the protein was not substantially affected by any of the SNPs, whereas cell surface expression and function of even double mutants showed no difference from the wild-type protein. The study suggests that these SNPs result in mutant proteins with a distribution and function similar to the wild-type protein. However, kinetic parameters for those variants have not been assessed in that study.

On the other hand, the ABCB1 2677G>T/A[64] contains a tri-allelic polymorphism (with G at nucleotide 2677 found in the wild-type sequence, and with A or T at that position being the two possible variants), which results in an amino acid change (A893S/T) in exon 21. Previous work has shown that the Ser893 substitution is associated with an altered drug resistance pattern in AdrR MCF-7 cells as well as enhanced efflux transporting ability in stably transfected NIH3T3 GP+E86 cells.[65]

2. FUNCTIONAL ANALYSIS OF POLYMORPHISM OF ABCB1

2.1. Effect of Nonsynonymous Polymorphisms on the Function of ABCB1 Figure 3 depicts hitherto identified nonsynonymous polymorphisms in the ABCB1 protein. Quantitative studies are required to precisely evaluate functional changes associated with genetic polymorphisms of ABCB1. For this purpose, the cDNA of ABCB1 was cloned from the human liver cDNA library, and several variant forms (*i.e.*, N21D, N44S, F103L, G185V, S400N, A893S, A893T, M986V) were prepared by site-directed mutagenesis (see Fig. 4A for primers). These variants and the wild type of ABCB1 were then expressed it in Sf9 cells using the pFASTBAC1 vector and recombinant baculoviruses. The expression of ABCB1 in Sf9 cells increased during the incubation. Three to 4 d after the infection, cells were harvested by centrifugation. ABCB1 variant proteins expressed in Sf9 cell membranes were detected by the western blot method using the C219 monoclonal antibody (Fig. 4B). Using membranes prepared from Sf9 cells expressing ABCB1 variants, ATPase activity was measured in the presence of verapamil at various concentrations. The ATPase activity of the isolated Sf9 cell membranes was determined by measuring inorganic phos-

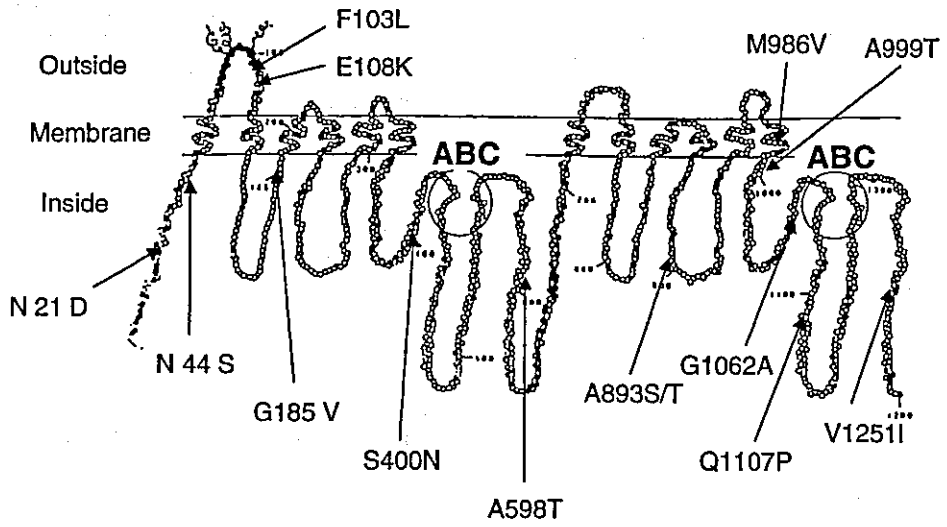

Fig. 3. Schematic Representation of ABCB1 and Nonsynonymous Polymorphism

The positions of amino acid substitutions are indicated by arrows. Data are from refs. 23—26 and 37 as well as the SNP database of NCBI. The molecular structure of ABCB1 is modified from Gottesman MM, Pastan I: The multidrug transporter, a double-edged sward. *J. Biol. Chem.*, 263, 12153—12166 (1988). ABC, ATP-binding cassette. (From ref. 16.)

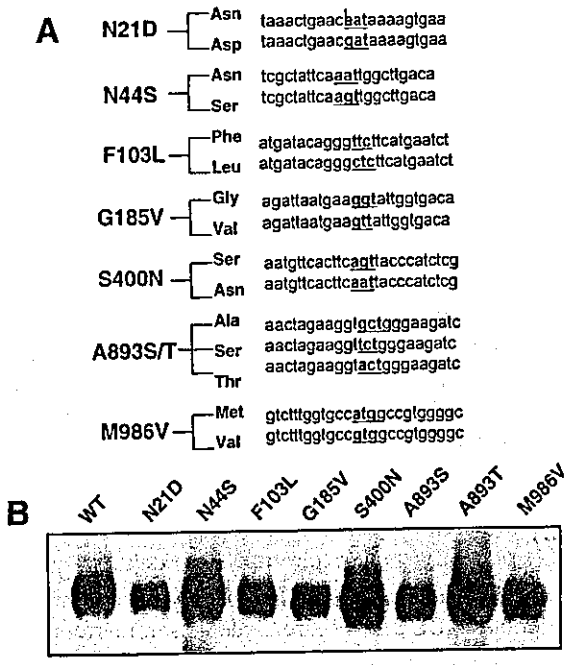

Fig. 4. Expression of ABCB1 Variants in Sf9 Cells

(A) PCR primers used for site-directed mutagenesis. The codons corresponding to amino acid substitutions are indicated by underlines. (B) Western blotting analysis for ABCB1 variants expressed in the plasma membrane fraction of Sf9 cells. (From refs. 16 and 71.)

Table 1. Kinetic Parameters of the Wild Type and SNP Variants of ABCB1

Variant	K_m (μM)	V_{max} (nmol/min/mg protein)
Wild type	2.190±0.150	13.14±1.95
N21D	0.502±0.126	45.26±11.33
N44S	0.580±0.148	31.03±4.65
F103L	1.100±0.078	36.34±8.33
G185V	0.831±0.102	56.76±6.76
S400N	0.327±0.025	13.74±2.08
A893S	0.441±0.042	17.24±6.72
A893T	0.904±0.244	10.77±1.35
M986V	0.419±0.062	22.69±6.84

The wild type and variants of ABCB1 were then expressed it in Sf9 cells using the pFASTBAC1 vector and recombinant baculoviruses. Using membranes prepared from Sf9 cells expressing ABCB1 variants, ATPase activity was measured in the presence of verapamil at different concentrations. K_m and V_{max} values were calculated from Lineweaver–Burk plots. ABCB1 proteins expressed in Sf9 cell membranes were detected by the western blot method using the C219 monoclonal antibody. The V_{max} values of the variants were normalized to that of the wild type by referring to the intensity of each variant protein on the western blotting. Data are expressed as mean values±S.D. (n=3).

phate liberation.[66,67] As demonstrated in Fig. 5, verapamil, one of the typical substrates of ABCB1, enhanced ATPase activity. Table 1 summarizes kinetic parameters observed for the variant forms as well as the wild type of ABCB1. The variant forms (*i.e.*, N21D, N44S, F103L, G185V, S400N, A893S, A893T, M986V) exhibited the verapamil-enhanced ATPase activity, as did the wild type of ABCB1. K_m values for verapamil were slightly different among those variants. The V_{max} values of the variants were normalized to that of the wild type by referring to the intensity of each variant protein on the western blotting (Fig. 4B). The variant G185V (acquired mutation) was found to have the highest V_{max} value, which was followed by N21D (Table 1). Thus, it is critically

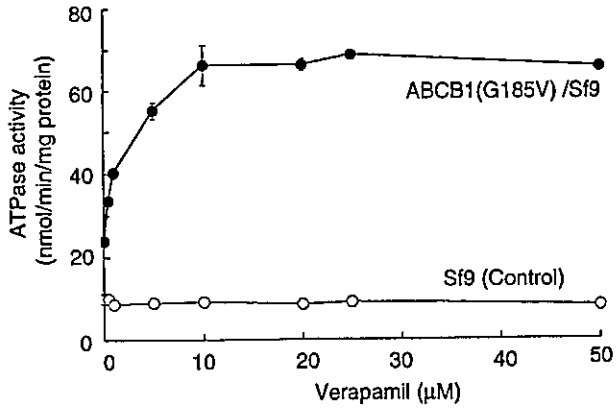

Fig. 5. Verapamil-Enhanced ATPase Activity in the Plasma Membrane Fraction of Sf9 Cells

Closed circles, ABCB1 (G185V)-expressing Sf9 cells; open circles, control Sf9 cells.

important to quantitatively analyze the functional difference among such variants in evaluating naturally occurring non-synonymous polymorphisms.

2.2. High-Speed Screening System to Analyze the Substrate Specificity of ABCB1 Since ABCB1 is an ATP-dependent active transporter, drug transport is coupled with ATP hydrolysis. There are two ATP-binding cassettes in one molecule of the ABCB1 protein. Those ATP-binding cassettes are functionally non-identical, but essential for the transport function of ABCB1.[68,69] ABCB1 ATPase activity is considered a functional probe for specific binding of transported drugs. It is possible to use this property to study the function and/or substrate specificity of ABCB1.[70] Based on the ABCB1 ATPase activity, we have developed a high-speed assay system using 96-well plates to analyze the substrate specificity of this activity.[71] Figure 6A schematically demonstrates the protocol of ATPase activity assay. Briefly, the Sf9 cell membranes expressing ABCB1 (2 μg of membrane protein per each well) were suspended in 10 μl of the incubation medium containing 50 mM Tris–Mes (pH 6.8), 2 mM EGTA, 2 mM dithiothreitol, 50 mM potassium chloride, 5 mM sodium azide, 2 mM ouabain. This medium was mixed with 10 μl of a test compound solution and then pre-incubated at 37 °C for 3 min. The ATPase reaction was started by adding 20 μl of 4 mM ATP/Mg solution to the reaction mixture and the incubation was maintained at 37 °C for 30 min. The reaction was stopped by the addition of 20 μl of 5% trichloroacetic acid and subsequently with 42 μl of "solution A" and "solution B" (see Fig. 6A for details). Thereafter, 120 μl of "solution C" was added to the mixture. These mixing processes were automatically carried out in the HALCS-I system (BioTec Co. Ltd., Tokyo Japan) (Fig. 6B). The absorbance of each reaction mixture in the 96-well plates was photometrically measured at a wavelength of 625 nm in a Multiskan JX system (Dainippon Pharmaceuticals Co., Osaka, Japan). The amount of liberated phosphate was quantified based on the calibration line established with inorganic phosphate standards (Fig. 6C). In addition, each 96-well plate contained a positive control, in which Sf9 cell membranes were incubated with 10 μM verapamil (Fig. 6C).

2.3. Comparison with Other Methods At present, there are several *in vitro* methods available for the screening

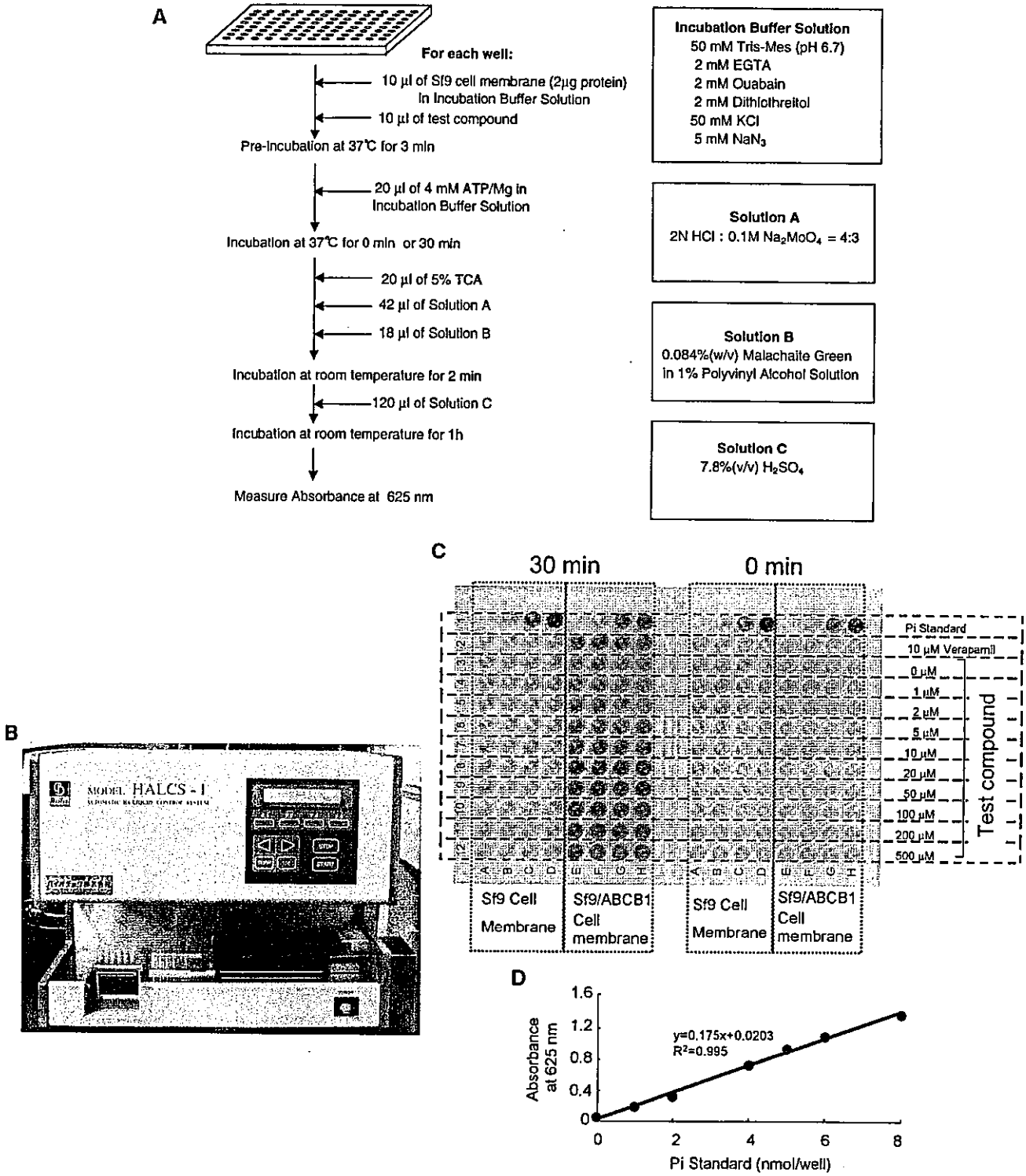

Fig. 6. High-Speed Screening of ABCB1 ATPase Activity

(A) Schematic diagram for the screening. (B) HALCS-I system (BioTec Co. Ltd., Tokyo Japan); (C) an example of 96-well plates prepared for ABCB1 ATPase assay. (From ref. 71.)

of drug compounds with regard to possible stimulation or inhibition of ABCB1 activity. A common method is to incubate the compound with cells that overexpress ABCB1 and then to measure the uptake in those cells after a defined interval. The same methodology can be used to study the inhibition of ABCB1-mediated transport by measuring the uptake of known substrates, such as verapamil and doxorubicin, with and without the compound in the media. Caco-2 cell monolayers are widely used in the pharmaceutical industry to assess membrane permeability and interaction with ABCB1 and other efflux transporters.[72] However, from a drug development perspective, those cellular assay methods are too costly and time-consuming. In addition, the cell density potentially involves a critical factor that makes the estimation of ABCB1-mediated transport difficult because of the paracellular transport of test compounds. Furthermore, there are

other intrinsic drug transporters expressed in Caco-2 cells. In this context, there is a considerable demand for rapid and efficient *in vitro* assay systems and computational methods to assess the biopharmaceutical properties of test compounds.

As compared with the Caco-2 cell system, the ATPase assay system developed in the present study is simple and makes it easy to measure the ATPase activity not only for ABCB1 but also for other ABC transporters, such as ABCG2 (BCRP/MXR1/ABCP).[73] In addition, Sf9 insect cells and recombinant baculoviruses are practical and cost-effective mesns to express ABCB1 or other ABC transporter proteins in large amounts, since Sf9 cells grow much faster than mammalian cells. As a consequence, ATPase measurements with ABCB1 expressend in Sf9 cell membranes are well suited for extensive studies involving a large number of molecules to be tested.

3. STRUCTURE–ACTIVITY RELATIONSHIP (SAR) ANALYSIS

3.1. Effect of Therapeutic Drugs on ABCB1 ATPase Activity Using the high-speed screening system, we have measured ABCB1 ATPase activity toward a total of 41 different therapeutic drugs and compounds. The tested compounds are classified into seven groups, *i.e.*, A, neurotransmitters; B, Ca^{2+} channel blockers, C, steroids; D, potassium channel modulators; D, non-steroidal anti-inflammatory drugs (NSAIDs); F, anti-cancer drugs; and G, miscellaneous. Figure 7 demonstrates the effects of those test compounds on ABCB1 ATPase activity. The concentration of test compounds was 10 μM in the measurement, and the data are expressed as relative values as compared with the ATPase activity measured with 10 μM verapamil. Among 41 different therapeutic drugs and compounds tested in this study, Ca^{2+} channel blockers, such as verapamil (B-1), bepridil (B-4), fendiline (B-5), prenylamine (B-6), and nicardipine (B-7), as well as FK506 (G-4) stimulated the ATPase activity. At the concentration of 100 μM, paclitaxel (F-5), doxorubicin (F-7), and quinidine (G-1) have more significantly stimulated the ABCB1 ATPase activity, whereas the extent of ATPase stimulation was relatively smaller than that of Ca^{2+} channel blockers (data not shown).

3.2. Correlation of the Substrate Specificity of ABCB1 and the Surface Activity of Test Compounds ABCB1 was originally assumed to function as a membrane pump for exporting intracellularly located substrates. However, it has recently been proposed that ABCB1 translocates a substrate from the inner leaflet side of the membrane to the outer leaflet side, thus, it functions as a flippase or membrane vacuum cleaner.[74] This mechanism is supported by the fact that most substrates of ABCB1 are hydrophobic compounds. Based on the latter mechanism, a substrate must be first dissolved in or adsorbed on the lipid bilayer of cellular membranes before they are recognized and subsequently transported by ABCB1. Therefore, the substrate specificity of ABCB1 is related to the lipophilicity and/or amphiphilicity of compounds.

While the lipophilicity is well characterized by the octanol/water partition coefficient (log P), Seelig *et al.* have devised a new method to predict amphiphilic properties of test compounds.[75,76] The criteria depend on the amphiphilic properties of a test compound as reflected in its surface activity. The surface activity is quantified by the Gibbs adsorption isotherms in terms of three parameters: (i) the onset of surface activity, (ii) the critical micelle concentration, and (iii) the surface area requirement of the test compound at the air/water interface.[75,76]

The adsorption of an amphiphile at the air/water interface lowers the surface tension of the buffer, γ_0, to a new value γ. The difference, $\pi=\gamma_0-\gamma$ is the so-called surface pressure. The thermodynamics of the absorption process is described by the Gibbs adsorption isotherm which can be written as

$$d\gamma=-RT(N_A A_S)^{-1} d\ln C=-RT\Gamma d\ln C=-d\pi \quad (1)$$

where C is the concentration of an amphiphilic compound in bulk solution, RT is the thermal energy, N_A is the Avogadro number and A_S is the surface area of the surface-active molecule at the interface. $\Gamma=(N_A A_S)^{-1}$ denotes the surface excess concentration. At low concentrations, Γ increases linearly with C; at high concentrations, Γ reaches a limiting value Γ^*. Thus, a plot of π *vs.* $\ln C$ should yield a straight line as long as Γ is constant. As was evaluated from the slope:

$$\Gamma^*=(1/RT)d\pi/d\ln C \quad (2)$$

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

$$\pi=RT\Gamma^*\ln(1+K_{aw}C) \quad (3)$$

where K_{aw} is the air/water partition coefficient. By fitting Eq. 3 to the measured π/C curve by using Γ^* determined according to Eq. 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 K_m 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 log P values.[77]

We have measured the surface activity of those 41 different compounds. Figure 8 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. 7. The two-dimensional plot of $\log K_{aw}$ values *vs.* ABCB1 ATPase activities (Fig. 8) 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.

3.3. 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 struc-

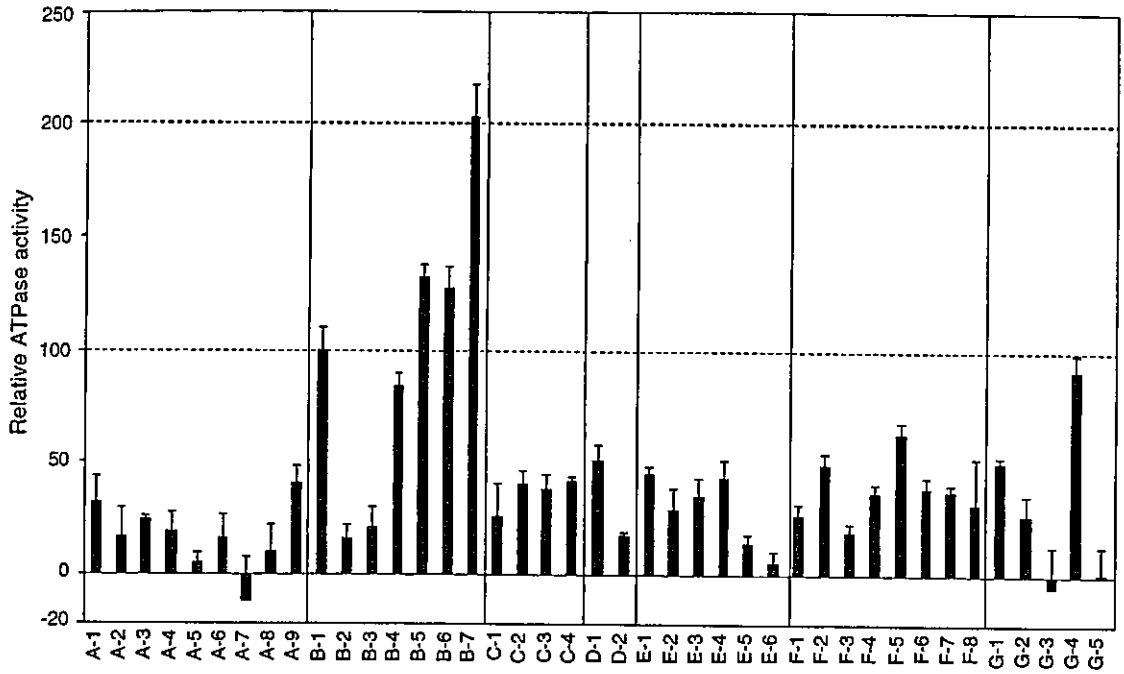

Fig. 7. The Effect of Therapeutic Drugs and Compounds on ABCB1 ATPase Activity

The ATPase activity was measured in the presence of 10 μM of a test compound. All the activities are expressed as relative values±S.D. as compared with the activity measured with 10 μM verapamil (100%). The tested drugs and compounds are: glycine (A-1), glutamic acid (A-2), dopamine (A-3), norepinephrine (A-4), epinephrine (A-5), γ-aminobutyric acid (A-6), histamine (A-7), serotonin (A-8), melatonin (A-9), verapamil (B-1), nifedipine (B-2), diltiazem (B-3), bepridil (B-4), fendiline (B-5), prenylamine (B-6), nicardipine (B-7), dexamethasone (C-1), betamethasone (C-2), prednisolone (C-3), cortisone (C-4), nicorandil (D-1), pinacidil (D-2), acetylsalicylic acid (E-1), indomethacin (E-2), acemetacin (E-3), ibuprofen (E-4), naproxen (E-5), mepirizole (E-6), vinblastine (F-1), etoposide (F-2), actinomycin D (F-3), daunorubicin (F-4), paclitaxel (F-5), methotrexate (F-6), doxorubicin (F-7), 5-fluorouracil (F-8), quinidine (G-1), *p*-aminohippuric acid (G-2), penicillin G (G-3), FK506 (G-4), and novobiocin (G-5). (From ref. 71.)

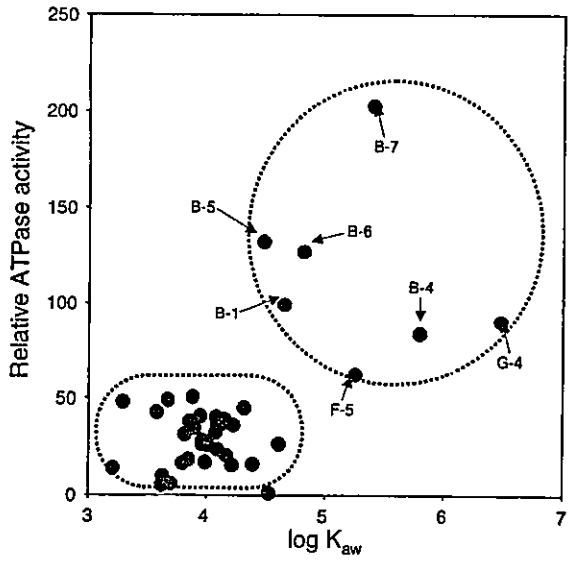

Fig. 8. Relationship between the ABCB1 ATPase Activities and the K_{aw} Values of the Therapeutic Drugs and Compounds Tested

The ATPase activities are expressed as relative values as compared with the activity measured with 10 μM verapamil (100%). (From ref. 71.)

tures 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 2 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. 9), where the predicted activity of the ABCB1 ATPase was well correlated with the observed ATPase activity. Table 3 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 3), suggesting that an oxo group bonded to an aliphatic carbon (Table 4) 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) 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

Table 2. 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.

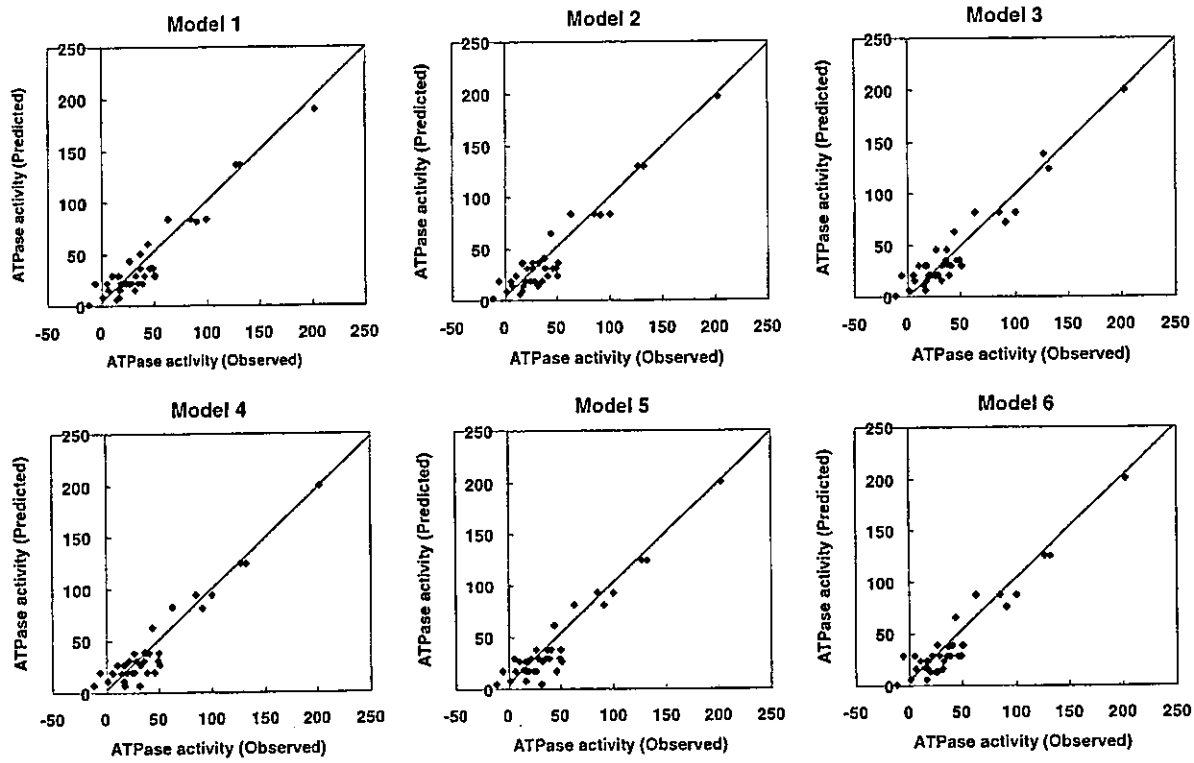

Fig. 9. Relationships between the Relative ATPase Activities Observed in Fig. 4 and the ATPase Activities Predicted from the Multiple Linear Regression Analysis. (From ref. 71.)

Table 3. 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 4. 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
M27	Chain bonded to U	M272	Chain bonded to O
M53	Carbocyclic systems with at least one aromatic ring	M531	One M53 code
F01	Positions substituted	F014	Position 4 substituted
H10	Type of amine	H100	One primary amine
M32	Multipliers for Subset M31 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.

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.[85] It would be of importance to further expand this 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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Review

Functional Evaluation of ABCB1 (P-Glycoprotein) Polymorphisms: High-Speed Screening and Structure-Activity Relationship Analyses

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Summary: Evidence is accumulating to strongly suggest that drug transporters are one of the determinant factors governing the pharmacokinetic profile of drugs. Effort has been made to identify genetic variation in drug transporter genes. In particular, genetic variations of the human *ABCB1* (*MDR1*) gene have been most extensively studied. Hitherto more than fifty single nucleotide polymorphisms (SNPs) and insertion/deletion polymorphisms in the *ABCB1* gene have been reported. However, at the present time, information is still limited with respect to the actual effect of those genetic polymorphisms on the function of ABCB1. In this context, we have undertaken functional analyses of ABCB1 polymorphisms. To quantify the impact of genetic polymorphisms on the substrate specificity of ABCB1, we have developed a high-speed screening system and a new structure-activity relationship (SAR) analysis method. This review addresses functional aspects of the genetic polymorphism of ABCB1 and provides the standard method to evaluate the effect of polymorphisms on the function.

Key words: ABC transporter; P-glycoprotein; MDR1; SNP; polymorphism; structure-activity relationship (SAR)

Introduction

Drug transporters and drug-metabolizing enzymes are important because they play pivotal roles in determining the pharmacokinetic profiles of drugs and, by extension, their overall pharmacological effects (i.e., drug absorption, drug distribution, drug metabolism and elimination, drug concentration at the target site, and the number and morphology of target receptors).[1-10] The effects of drug transporters on the pharmacokinetic profile of a drug depend on their expression and functionality. Indeed, the expression of drug transporters can be modulated by endogenous and exogenous factors, including drugs, themselves. It is also now known that inherited differences among individuals may also affect drug efficacy and toxicity.[11-13] Such inherited differences include genetic polymorphisms in drug targets and drug-metabolizing enzymes, as well as in drug transporters. Hitherto, pharmacogenetics, the field dealing with such inherited differences and their effect on pharmacokinetics, has significantly contributed to our understanding of genetic causes underlying differences in drug metabolism (e.g., cytochrome P-450 mediated drug metabolism). In fact, recent technological advances allowing massive molecular sequencing have in turn allowed us to identify single nucleotide polymorphisms (SNPs) as one possible cause of variable drug response among individuals.[14,15] In light of such advances, it is important to carefully examine the clinical significance, if any, of polymorphisms in drug response genes, including drug transporters.

There are an increasing number of literatures that address genetic polymorphisms of drug transporters. At present, concerning the effects of genetic polymorphisms on pharmacokinetic profiles, the best characterized transporter is ABCB1. However, it is also true that there is still considerable discrepancy among hitherto reported results. There are many factors that can affect the function as well as the expression of drug transporters. Those factors may involve genetic mutations, SNPs, splicing, transcriptional regulation, stability of mRNA, post-translational modification, and intracellular localization. Evaluation of such factors is critically important to understand the whole picture of pharamacogenomics of drug transporters. Functional analysis of the polymorphism of drug transporters is

Received; December 17, 2003, Accepted; January 13, 2004
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one of such important approaches that provide clear insight into the biochemical significance of genetic polymorphisms.[16] 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. The present review article provides a new approach of quantitative analysis for the genetic polymorphism of ABCB1 (P-glycoprotein or MDR1) and its functionality.

ABCB1 (P-glycoprotein/MDR1)

Human ABCB1 (P-glycoprotein or MDR1) was identified because of its overexpression in cultured cancer cells associated with an acquired cross-resistance to multiple anticancer drugs.[17] While "P-glycoprotein" was initially thought to play a role in modulating cellular permeability ('P' stands for permeability) to drugs, it has later been demonstrated to be an ATP-dependent efflux pump of hydrophobic anticancer drugs including colchitine, doxorubicin, daunorubicin, vincristiene and VP16. Historically P-glycoprotein provided one of the mechanistic explanations for the multidrug resistance phenomeon. The function of ABCB1 as a mechanism of multidrug resistance has been extensively investigated.[18]

Molecular structure: Human ABCB1 and its orthologues in mammalians are single peptide chains, integral membrane proteins of an approximate length of 1280 amino acid residues. The apparent molecular weight of mature ABCB1 ranges from about 130 to 180 kDa, depending on the species and cell type in which they are expressed. ABCB1 is composed of two homologous halves each of which consist of an N-terminal, hydrophobic, membrane-associated domain (approximately 250 amino acid residues) and a C-terminal, hydrophilic nucleotide binding fold (approximately 300 amino acid residues). The plasma membrane associated domains in the two halves of ABCB1 each consist of six transmembrane domains, which are followed by an intracellular ATP-binding cassette.

In order to elucidate the transport mechanism and structure of ABCB1, mutational analysis was widely carried out using site-directed mutagenesis. A relatively large number of mutants alter the transporter's substrate specificity, in particular those in transmembrane domains 5, 6, and 12. Accordingly, photo-affinity labeling studies indicated that such domains probably are of major importance in substrate binding.

Gene structure: Humans *ABCB1* and *ABCB4* genes are adjacently located on chromosome 7q21. *ABCB1* encodes a drug transporter directly associated multidrug resistance of cancer, whereas *ABCB4* encodes the flippase translocating phopholipids. In rodents, three genes are present: *mdr1a*, *mdr1b*, and *mdr2*. In the mouse, the genes are clustered on chromosome 5 whereas they are located at chromosomal region 4q11-12 in the rat. In rodents, both Mdr1a and Mdr1b functionally correspond to ABCB1 (MDR1) in humans.

Transcription of the *ABCB1* gene appears to be regulated by multiple factors. For example, the proximal promoter region has a GC-rich region, at approximately −100 to −120 bp from the transcriptional start codon, that contains a site responsible for the repression of transcription. Also, basal transcription appears to involve a consensus site that binds NF-Y transcription factors at a Y-box (inverted CCAAT box) between −70 and −80 bp. In addition, a binding site for SP-1 and members of the early growth response (EGR) family of transcriptional factors is present which overlaps with the NF-Y consensus site. A 13-bp region around the initiation site involved in accurate initiation of the transcription has also been identified. ABCB1 is expressed at a high frequency in tumor cells and both c-H-Ras and mutant forms of p53 have been shown to activate the ABCB1 promoter. On the other hand, c- and N-Myc expression is apparently inversely correlated with ABCB1 expression. In addition to such transcriptional regulations, the stability of mRNA and posttranslational regulation are also considered important in the regulation of ABCB1 expression.

ABCB1 in normal tissues: It is important to know that ABCB1 is expressed not only in cancer cells but also in many normal tissues. For example, it is located in the apical domain of the enterocytes of the gastrointestinal tract (jejunum and duodenum) and limits the uptake and absorption of drugs and other substrates from the intestine into the systemic circulation by excreting substrates into the gastrointestinal tract. In addition, ABCB1 is expressed in endothelial cells lining the small vessels of the human cortex, in which the transporter appears concentrated within the lumenal cellular compartment.[19] The expression of ABCB1 on the lumenal membrane of capillary endothelial cells of the brain restricts drug distribution into the central nervous system. This function of ABCB1 appears to be very important in protecting the central nervous system from the attack of toxic compounds. Evidence for the protective role of ABCB1 in the blood-brain barrier has been demonstrated in several studies using *mdr1a* knockout mice.[20] A similar protective role to limit the distribution of potentially toxic xenobiotics into tissues was suggested for ABCB1 expressed in the placenta and the testis. ABCB1 expressed in the canalicular domain of the hepatocyte and the brush border of the proximal renal tubule plays a role in the biliary and urinary excretion of xenobiotics and endogenous compounds.

Acquired mutation of ABCB1 in cancer cells: Cells selected *in vitro* against a lipophilic cytotoxic compound usually develop cross resistance to other drugs. Some multidrug-resistant cell lines are significantly more resistant to the drug used in their selection than to the

Table 1. Effect of SNP 3435C>T of *ABCB1* on pharmacokinetic profile

Drug	Population (CC/CT/TT)	Pharmacokinetic parameter	Effects	Reference
Cyclosporin	RT recipients (31/52/41)	Trough levels	N.S.	49
Cycrosporin	Volunteers (7/6/1)	AUC, C_{max}	N.S.	50
Cycrosporin	HT recipients (14 total)	AUC, C_{max}	N.S.	51
Cycrosporin	RT recipients (108 total)	Trough levels	N.S.	52
Dicloxacillin	Volunteers (17 total)	Clearance, C_{max}	N.S.	53
Digoxin	Volunteers (3/4/1)	AUC (rifampin induction)	Significantly higher with T allele	38
Digoxin	Volunteers (7/0/7)	C_{max}	Significantly higher in TT	38
Digoxin	Volunteers (5/4/3)	AUC (0–4 and 0–24 h)	N.S.	54
Digoxin	Volunteers (5/4/4)	AUC (0–4 h)	Significantly lower in TT or CT	55
Digoxin	Volunteers (50 total)	AUC, C_{max}	N.S.	56
Digoxin	Volunteers (8/8/8)	AUC (0–4 h), C_{max}	Significantly higher in TT	33
Dugoxin	Volunteers (5/5/5)[c]	F	Significantly higher in TT	57
Digoxin	Volunteers (6/0/6)	AUC (0–4 and 0–24 h)	Significantly higher in TT	58
Docetaxel	Cancer patients (5/16/7)	Clearance	N.S.	59
Fexofenadine	Volunteers (9/16/12)	AUC (0–4 h)	Significantly lower in TT	35
Fexofenadine	Volunteers (10/0/10)	AUC, clearance	N.S.	42
Irinotecan	Cancer patients (16/35/8)	AUC, clearance	N.S.	23
Midazolam	Cancer patients (5/16/7)	Clearance	N.S.	59
Nelfinavir	NIV-1 patients (10/39/14)	Plasma concentrations	Significantly lower with T allele	44
Nortriptyline	Depressed patients (78 total)	Postural hypotension	Risk increased in TT	36
Phenytoin	Volunteers (28/45/23)	Plasma concentrations	Lower in CC	22
Tacrolimus	LT recipients (15/22/9)	Concentration/dose ratio	N.S.	60
Tacrolimus	RT recipients (48/70/62)	Plasma concentrations	Lower in CC	32
Tacrolimus	LT recipients (4/10/3)	Neurotoxicity	N.S.	60
Tacrolimus	RT recipients (62 total)	Trough concentrations	N.S.	52
Talinolol	Volunteers (13/29/13)	AUC, F, C_{max}	N.S.	34
Tipifarnib	Cancer patients (29 total)	AUC	N.S.	62

Abbreviations: CC, wild-type; CT, heterozygous variant; TT, homozygous variant; AUC, area under the time-concentration curve; RT, renal transplant; HT, heart transplant; LT, liver transplant; C_{max}, maximum concentration; F, oral bioavailability; N.S., no significant difference.

other drugs. A single amino acid substitution, Gly185Val, in the human ABCB1 protein was found to cause an altered pattern of drug resistance in cell lines transfected with the ABCB1 cDNA carrying this mutation.[21] It is suggested that the amino acid at position 185 is involved in colchicines and verapamil but not in vinblastine binding/transport. In addition, several recombinant variants have been generated either by *in vivo* drug selection or by site-directed mutagenesis techniques, which show altered substrate specificity or impaired function of a properly assembled protein.[18]

Naturally occurring SNPs of ABCB1: To date, genetic variations of the human *ABCB1* gene have been most extensively studied (ref. 22, 23 for recent reviews). Hitherto 50 SNPs and 3 insertion/deletion polymorphisms in the *ABCB1* gene have been reported.[24-27] In addition, twelve novel SNPs of ABCB1 were reported in Japanese patients with ventricular tachycardia who were administered amiodarone.[28] Several preclinical and clinical studies have provided evidence for the naturally occurring polymorphisms in ABCB1 and their effects on drug absorption, distribution and elimination[29-62] (**Table 1**). Hoffmeyer *et al.* reported multiple polymorphisms in the *ABCB1* gene.[38] One of those mutations in particular, a C-to-T variant at position 3435 in the exon 26 of the *ABCB1* gene, was reportedly correlated with ABCB1 expression and function. While this SNP does not alter the amino acid sequence of ABCB1, individuals homozygous for the polymorphism expressed significantly less duodenal ABCB1 and significantly more plasma digoxin.[38] The distribution of the SNP C3435T in exon 26 in the Chinese and Malay population was found to be similar to the Caucasians whereas the Indians were different. The Asian population also differed significantly from the African and Caucasian population in the distribution of the C3435T SNP.[39,40] However, the association of the C3435T polymorphism with ABCB1 protein expression and function remains controversial. In fact, various investigators have reported that the T allele is associated with increased (or decreased) expression levels or has no clearly discernible effect.[41-48] Furthermore, the reported effects of C3435T SNP on the pharmacokinetic profiles of drugs are also controversial (see **Table 1**).[49-62]

Recently, five most common coding SNPs were assessed by a vaccinia virus-based transient expression system.[63] The study on cellular accumulation of several tested substrates indicated that the substrate specificity of the protein was not substantially affected by any of the SNPs, whereas cell surface expression and function

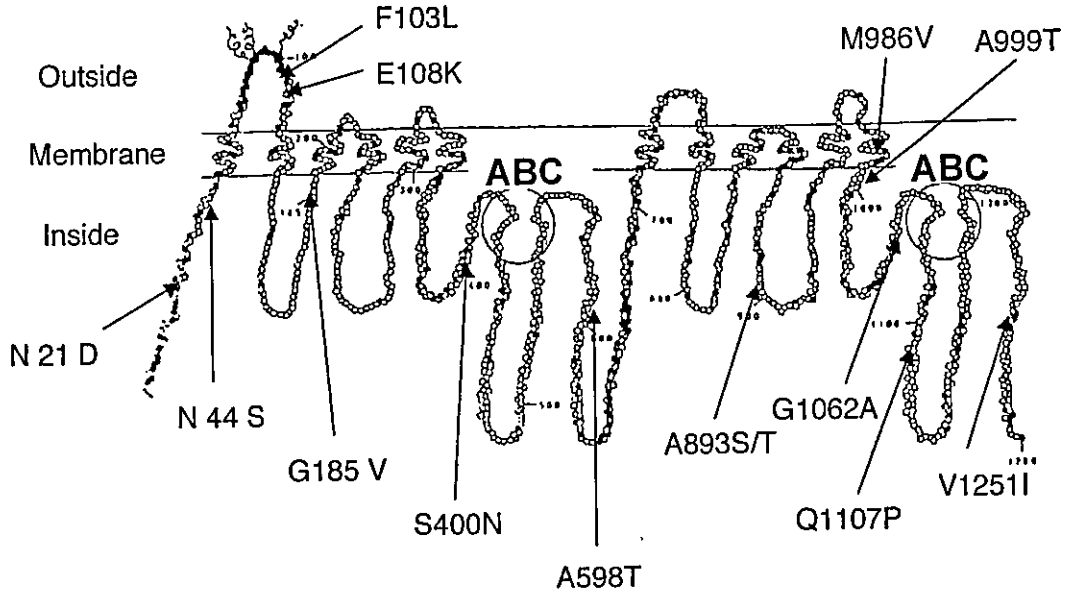

Fig. 1. Schematic representation of ABCB1 and nonsynonymous polymorphism. The positions of amino acid substitutions are indicated by arrows. Data are from refs. 23–26 and 37 as well as the SNP database of NCBI. The molecular structure of ABCB1 is modified from Gottesman MM, Pastan I: The multidrug transporter, a double-edged sward. *J. Biol. Chem.* 263, 12153–12166 (1988). ABC, ATP-binding cassette. (From ref. 16)

of even double mutants showed no difference from the wild-type protein. The study suggests that these SNPs result in mutant proteins with a distribution and function similar to the wild-type protein. However, kinetic parameters for those variants have not been assessed in that study.

On the other hand, the ABCB1 G2677T/A[64] contains a tri-allelic polymorphism (with G at nucleotide 2677 found in the wild-type sequence, and with A or T at that position being the two possible variants), which results in an amino acid change (A893S/T) in exon 21. Previous work has shown that the Ser893 substitution is associated with an altered drug resistance pattern in AdrR MCF-7 cells as well as enhanced efflux transporting ability in stably transfected NIH3T3 GP+E86 cells.[65]

Functional analysis of polymorphism of ABCB1: **Figure 1** depicts hitherto identified nonsynonymous polymorphisms in the ABCB1 protein. Quantitative studies are required to precisely evaluate functional changes associated with genetic polymorphisms of ABCB1. For this purpose, the cDNA of ABCB1 was cloned from the human liver cDNA library, and several variant forms (i.e., N21D, N44S, F103L, G185V, S400N, A893S, A893T, M986V) were prepared by site-directed mutagenesis (see **Fig. 2A** for primers). These variants and the wild type of ABCB1 were then expressed it in Sf9 cells using the pFASTBAC1 vector (**Fig. 2B**) and recombinant baculoviruses. The expression of ABCB1 in Sf9 cells increased during the incubation (**Fig. 2C**). Three to 4 days after the infection, cells were harvested by centrifugation. ABCB1 variant proteins expressed in Sf9 cell membranes were detected by the western blot method using the C219 monoclonal antibody (**Fig. 2D**). Using membranes prepared from Sf9 cells expressing ABCB1 variants, ATPase activity was measured in the presence of verapamil at various concentrations. The ATPase activity of the isolated Sf9 cell membranes was determined by measuring inorganic phosphate liberation[66] according to the procedure reported by Sarkadi *et al.*[67] with some modifications.

Table 2 summarizes kinetic parameters observed for the variant forms as well as the wild type of ABCB1. The variant forms (i.e., N21D, N44S, F103L, G185V, S400N, A893S, A893T, M986V) exhibited the verapamil-enhanced ATPase activity, as did the wild type of ABCB1. Km values for verapamil were slightly different among those variants. The Vmax values of the variants were normalized to that of the wild type by referring to the intensity of each variant protein on the western blotting (**Fig. 2D**). The variant G185V (acquired mutation) was found to have the highest Vmax value, which was followed by N21D (**Table 2**). Thus, it is critically important to quantitatively analyze the functional difference among such variants in evaluating naturally occurring nonsynonymous polymorphisms.

High-speed screening system to analyze the substrate specificity of ABCB1: Since ABCB1 is an ATP-dependent active transporter, drug transport is coupled with ATP hydrolysis. There are two ATP-binding cassettes in one molecule of the ABCB1 protein. Those ATP-binding cassettes are functionally non-identical, but

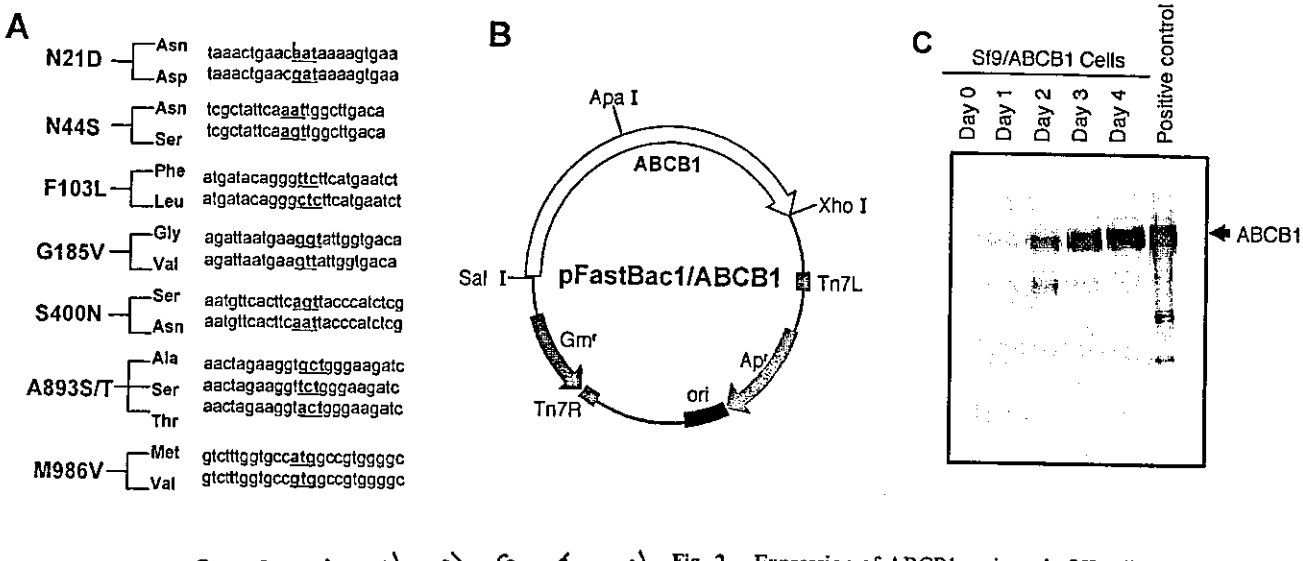

Fig. 2. Expression of ABCB1 variants in Sf9 cells. **A.** PCR primers used for site-directed mutagenesis. The sequences are compared with those of the wild type ABCB1. The codons corresponding to amino acid substitutions are indicated by underlines. **B,** pFASTBAC vector encoding the cDNA of human ABCB1 (P-glycoprotein). **C,** Expression levels of ABCB1 in Sf9 cell membrane. The plasma membrane was prepared from Sf9 cells after infection with recombinant baculoviruses on the days indicated. ABCB1 protein was detected by western blot. The positive control was ABCB1 expressed in Sf9 cell membranes (BD Gentest, Woburn, MA, USA). **D,** Western blotting analysis for ABCB1 variants expressed in the plasma membrane fraction of Sf9 cells. (From ref. 16 and 71)

Table 2. Kinetic parameters of the wild type and SNP variants of ABCB1

Variant	Km	Vmax
	(μM)	(nmol/min/mg protein)
Wild type	2.190±0.150	13.14±1.95
N21D	0.502±0.126	45.26±11.33
N44S	0.580±0.148	31.03±4.65
F103L	1.100±0.078	36.34±8.33
G185V	0.831±0.102	56.76±6.76
S400N	0.327±0.025	13.74±2.08
A893S	0.441±0.042	17.24±6.72
A893T	0.904±0.244	10.77±1.35
M986V	0.419±0.062	22.69±6.84

The wild type and variants of ABCB1 were then expressed it in Sf9 cells using the pFASTBAC1 vector and recombinant baculoviruses. Using membranes prepared from Sf9 cells expressing ABCB1 variants, ATPase activity was measured in the presence of verapamil at different concentrations. Km and Vmax values were calculated from Lineweaver-Burk plots. ABCB1 proteins expressed in Sf9 cell membranes were detected by the western blot method using the C219 monoclonal antibody. The Vmax values of the variants were normalized to that of the wild type by referring to the intensity of each variant protein on the western blotting. Data are expressed as mean values ±S.D. (n=3).

essential for the transport function of ABCB1.[68,69] ABCB1 ATPase activity is considered a functional probe for specific binding of transported drugs. It is possible to use this property to study the function and/or substrate specificity of ABCB1.[70] Based on the ABCB1 ATPase activity, we have developed a high-speed assay system using 96-well plates to analyze the substrate specificity of this activity.[71] **Figure 3** schematically demonstrates the protocol of ATPase activity assay. Briefly, the Sf9 cell membranes expressing ABCB1 (2 μg of membrane protein per each well) were suspended in 10 μL of the incubation medium containing 50 mM Tris-Mes (pH 6.8), 2 mM EGTA, 2 mM dithiothreitol, 50 mM potassium chloride, 5 mM sodium azide, 2 mM ouabain. This medium was mixed with 10 μL of a test compound solution and then pre-incubated at 37°C for 3 min. The ATPase reaction was started by adding 20 μL of 4 mM ATP solution to the reaction mixture (40 μL) and the incubation was maintained at 37°C for 30 min. The reaction was stopped by the addition of 20 μL of 5% trichloroacetic acid and liberated inorganic phosphate was measured at wavelength of 630 nm[66] in a Multiskan JX system (Dainippon Pharmaceuticals Co., Osaka, Japan).

At present, there are several *in vitro* methods available for the screening of drug compounds with regard to

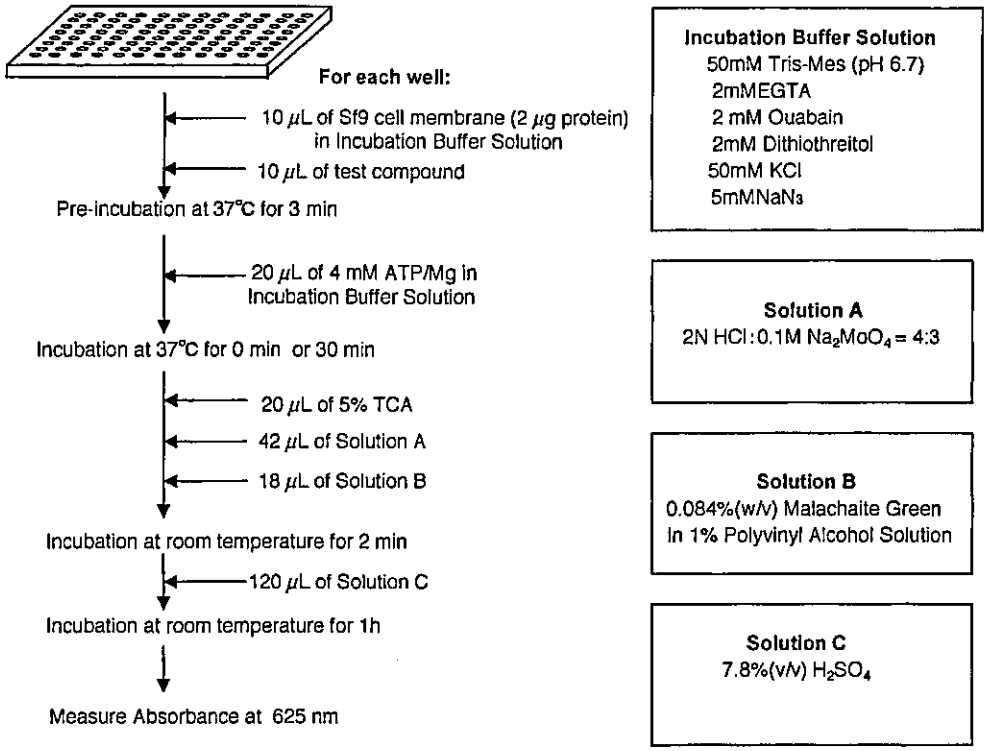

Fig. 3. Schematic diagram for the high-speed screening of ABCB1 ATPase activity. (From ref. 71)

possible stimulation or inhibition of ABCB1 activity. A common method is to incubate the compound with cells that overexpress ABCB1 and then to measure the uptake in those cells after a defined interval. The same methodology can be used to study the inhibition of ABCB1-mediated transport by measuring the uptake of known substrates, such as verapamil and doxorubicin, with and without the compound in the media. Caco-2 cell monolayers are widely used in the pharmaceutical industry to assess membrane permeability and interaction with ABCB1 and other efflux transporters.[72] However, from a drug development perspective, those cellular assay methods are too costly and time-consuming. In addition, the cell density potentially involves a critical factor that makes the estimation of ABCB1-mediated transport difficult because of the para-cellular transport of test compounds. Furthermore, there are other intrinsic drug transporters expressed in Caco-2 cells. In this context, there is a considerable demand for rapid and efficient *in vitro* assay systems and computational methods to assess the biopharmaceutical properties of test compounds.

As compared with the Caco-2 cell system, the ATPase assay system developed in the present study is simple and makes it easy to measure the ATPase activity not only for ABCB1 but also for other ABC transporters, such as ABCG2 (BCRP/MXR1/ABCP).[73] In addition, Sf9 insect cells and recombinant baculoviruses are practical and cost-effective mesns to express ABCB1 or other ABC transporter proteins in large amounts, since Sf9 cells grow much faster than mammalian cells. As a consequence, ATPase measurements with ABCB1 expressend in Sf9 cell membranes are well suited for extensive studies involving a large number of molecules to be tested.

Structure-Activity Relationship (SAR) analysis

Effect of therapeutic drugs on ABCB1 ATPase activity: Using the high-speed screening system, we have measured ABCB1 ATPase activity toward a total of 41 different therapeutic drugs and compounds. The tested compounds are classified into seven groups, *i.e.*, A, neurotransmitters; B, Ca^{2+} channel blockers, C, steroids; D, potassium channel modulators; D, non-steroidal anti-inflammatory drugs (NSAIDs); F, anti-cancer drugs; and G, miscellaneous. **Figure 4** demonstrates the effects of those test compounds on ABCB1 ATPase activity. The concentration of test compounds was 10 μM in the measurement, and the data are expressed as relative values as compared with the ATPase activity measured with 10 μM verapamil. Among 41 different therapeutic drugs and compounds tested in this study, Ca^{2+} channel blockers, such as verapamil (B-1), bepridil (B-4), fendiline (B-5), prenylamine (B-6), nicardipine (B-7), and FK506 (G-4) stimulated the ATPase activity. At the concentration of

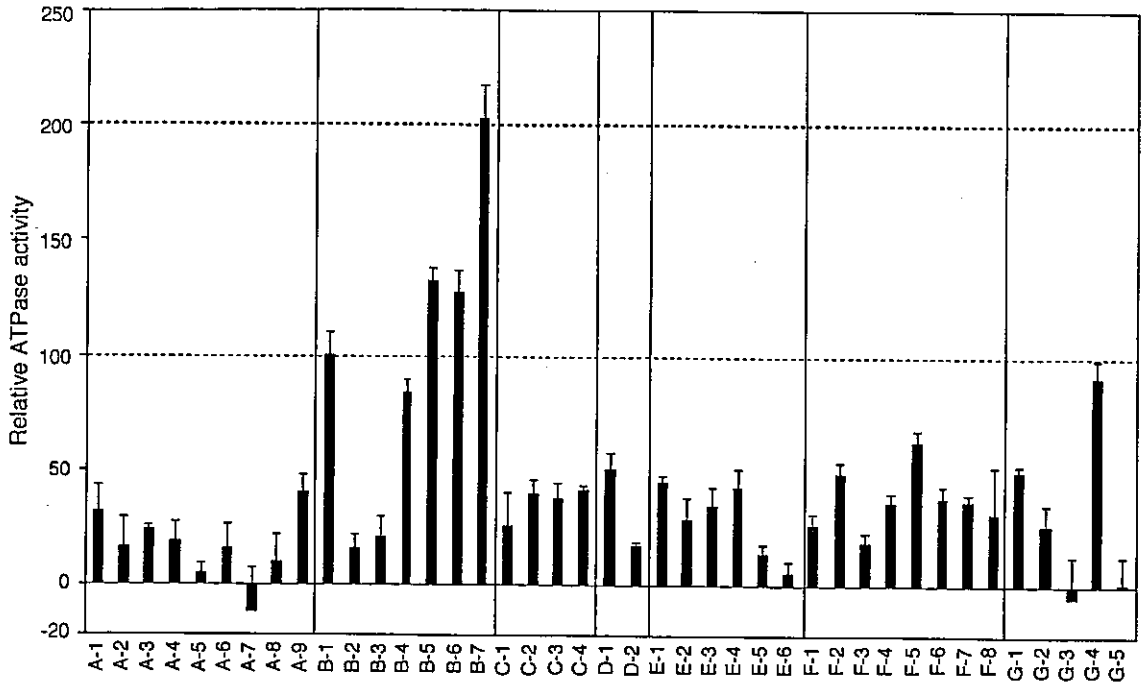

Fig. 4. The effect of therapeutic drugs and compounds on ABCB1 ATPase activity. The ATPase activity was measured in the presence of 10 μM of a test compound. All the activities are expressed as relative values ± S.D. as compared with the activity measured with 10 μM verapamil (100%). The tested drugs and compounds are: glycine (A-1), glutamic acid (A-2), dopamine (A-3), norepinephrine (A-4), epinephrine (A-5), γ-aminobutyric acid (A-6), histamine (A-7), serotonin (A-8), melatonin (A-9), verapamil (B-1), nifedipine (B-2), diltiazem (B-3), bepridil (B-4), fendiline (B-5), prenylamine (B-6), nicardipine (B-7), dexamethasone (C-1), betamethasone (C-2), prednisolone (C-3), cortisone (C-4), nicorandil (D-1), pinacidil (D-2), acetylsalicylic acid (E-1), indomethacin (E-2), acemetacin (E-3), ibuprofen (E-4), naproxen (E-5), mepirizole (E-6), vinblastine (F-1), etoposide (F-2), actinomycin D (F-3), daunorubicin (F-4), paclitaxel (F-5), methotrexate (F-6), doxorubicin (F-7), 5-fluorouracil (F-8), quinidine (G-1), *p*-aminohippuric acid (G-2), penicillin G (G-3), FK506 (G-4), and novobiocin (G-5). (From ref. 71)

100 μM, paclitaxel (F-5), doxorubicin (F-7), and quinidine (G-1) have more significantly stimulated the ABCB1 ATPase activity, whereas the extent of ATPase stimulation was relatively smaller than that of Ca^{2+} channel blockers (data not shown).

Correlation of the substrate specificity of ABCB1 and the surface activity of test compounds: ABCB1 was originally assumed to function as a membrane pump for exporting intracellularly located substrates. However, it has recently been proposed that ABCB1 translocates a substrate from the inner leaflet side of the membrane to the outer leaflet side, thus, it functions as a flippase or membrane vacuum cleaner.[74] This mechanism is supported by the fact that most substrates of ABCB1 are hydrophobic compounds. Based on the latter mechanism, a substrate must be first dissolved in or adsorbed on the lipid bilayer of cellular membranes before they are recognized and subsequently transported by ABCB1. Therefore, the substrate specificity of ABCB1 is related to the lipophilicity and/or amphiphilicity of compounds.

While the lipophilicity is well characterized by the octanol/water partition coefficient (logP), Seelig *et al.* have devised a new method to predict amphiphilic properties of test compounds.[75,76] The criteria depend on the amphiphilic properties of a test compound as reflected in its surface activity. The surface activity is quantified by the Gibbs adsorption isotherms in terms of three parameters: (i) the onset of surface activity, (ii) the critical micelle concentration, and (iii) the surface area requirement of the test compound at the air/water interface.[75,76]

The adsorption of an amphiphile at the air/water interface lowers the surface tension of the buffer, γ_0, to a new value γ. The difference, $\pi = \gamma_0 - \gamma$ is the so-called surface pressure. The thermodynamics of the absorption process is described by the Gibbs adsorption isotherm which can be written as

$$dy = -RT(N_A A_S)^{-1}\, dlnC = -RT\Gamma dlnC = -d\pi \qquad \text{Equation (1),}$$

where C is the concentration of an amphiphilic compound in bulk solution, RT is the thermal energy, N_A is the Avogadro number and A_S is the surface area of the surface-active molecule at the interface. $\Gamma = (N_A A_S)^{-1}$ denotes the surface excess concentration. At low concentrations, Γ increases linearly with C; at high concentrations, Γ reaches a limiting value Γ^*. Thus, a plot of π vs. lnC should yield a straight line as long as Γ is constant. As was evaluated from the slope: