Inhibitors of Multidrug Efflux Transporters: Their Membrane and Protein Interactions

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Abstract: Modulators and inhibitors of multidrug efflux transporters, like P-glycoprotein, are used to reduce or inhibit multidrug resistance, MDR, which leads to a failure of the chemotherapy of e.g. cancers, epilepsy, bacterial, parasitic, and fungal diseases. Binding and transport of first-, second-, and third-generation modulators and inhibitors of P-glycoprotein are discussed, taking into account the properties of the drug (H-bonding potential, dimensions, and pK_a values) as well as the properties of the membrane.

Keywords: P-glycoprotein, first-, second-, and third-generation inhibitors, modulators, membrane partitioning, transporter binding, transport kinetics.

INTRODUCTION

ATP binding cassette, ABC, efflux transporters are phylogenetically old proteins, which are present in prokaryotes [1, 2] and eukaryotes [3-5]. They protect cells against a wide variety of chemically unrelated toxins and drugs, which can cross the cell membrane by passive diffusion in the absence of transporters.

The first drug efflux pumps found to contribute to multidrug resistance, MDR, were P-glycoprotein, P-gp, in cancer cells [6] and a tetracycline exporting transporter in *E. coli* [7]. The same stimuli that induce MDR in humans also induce multidrug resistance in bacteria, parasites, and fungi, which express efflux transporter related to P-gp. MDR is a matter of growing concern not only in chemotherapy of cancers [8], but also in chemotherapy of epilepsy [9], bacterial [10], parasitic [11] and fungal [12] diseases.

Since P-gp is the best investigated representative of this class of transporters (for review see [13]) it will serve as a model for other efflux transporters of lipid soluble compounds. P-gp binds its substrates in the cytosolic membrane leaflet [14] most likely via H-bond formation [15] and moves them to the extracellular leaflet [16] or to the extracellular aqueous environment [17] at the expense of one [18-20] to two [21] molecules of ATP. Since re-equilibration of drugs between the extracellular solution and the outer membrane leaflet is fast an unambiguous differentiation between a flip-flop [22] and a transport mechanism [23] is difficult. In the absence of exogeneous substrates P-gp shows basal activity which may be due either to uncoupled ATPase activity [24], or to the transport of endogeneous substrates e.g. lipids [25].

The expression level of P-gp is high in tissues with protective barrier functions such as the brush border of the proximal tubules of the kidney, the bile canicular membrane of hepatocytes, the apical membrane of mucosal cells in the intestine, the luminal surface of the secretory epithelium of the gravid uterus and the luminal membrane of endothelial cells at the blood-brain barrier sites [26]. High expression levels are also found in cancers [27]. In a given tissue the expression level of P-gp and other efflux transporters can vary depending on genetic predisposition [28], age [29], diet and medication [30, 31]. Cells which express the MDR phenotype can over-express P-gp after exposure to a single agent (e.g. cytotoxic anticancer drugs, certain antibiotics or food components, with characteristic H-bond acceptor patterns) [32] or to physical stress, such as X-ray [33], UV light irradiation [34] and heat shock [35]. As a result cells become resistant not only to the resistance-inducing compound, but to all other compounds that are substrates for the efflux transporters being overexpressed. This phenomenon called MDR hampers chemotherapy of cells or tissues protected by efflux transporters.

To date, most strategies for reversing MDR have focused on modulation or inhibition of P-gp activity [13, 27], which can be achieved by (i) an inhibition of ATP binding, ATP hydrolysis or coupling of ATP hydrolysis to the translocation of substrates (e.g. azido-ATP, NBD chloride) [36, 37], (ii) inhibition of conformational changes required for drug extrusion via antibody binding to certain extracellular loops of the transporter (e.g. antibodies MRK-16 and UIC2) [38-40], and (iii) by non-competitive or competitive inhibition of P-gp achieved by direct interaction of a compound with one or more binding sites on P-gp (cf. e.g. [41]).

Non-competitive and competitive modulators and inhibitors of P-gp are in clinical trial (cf. Table **2**) and are co-administered with the therapeutically relevant drug to prevent or reduce an export of the latter. The first compounds found to act as modulators were commonly used drugs (first-generation inhibitors) belonging to many different therapeutic categories [41-45]. In general, these drugs are substrates at low concentrations and modulators at high concentrations [46, 47]. Since they have other therapeutic targets than P-gp they can show severe side effects at the high concentrations required for modulation. A second generation of modulators and inhibitors has therefore been designed based on stereoisomers and analogs of first-generation inhibitors. They have no therapeutic targets other than P-gp and are thus more selective and less toxic.

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Second-generation modulators and the most efficient first-generation modulators like e.g. PSC 833 and cyclosporin A, respectively, show, however, pharmacokinetic interactions with other drugs [48]. This is due to the fact that these compounds also reduce the activity of cytochrome P450, which shares overlapping substrate specificity with P-gp [49], and in turn enhance the general toxicity of co-administered drugs (e.g. anticancer drugs).

A third generation of inhibitors with enhanced selectivity, limited toxicity as well as limited pharmacokinetic interactions with other drugs [50, 51] is now in clinical trial. Recently, it has been realized that detergent-like compounds, which are often used as solubility enhancers can also modulate P-gp ([52-54], Nervi and Seelig, in preparation).

To develop safe and efficient inhibitors, the reversal mechanisms must be understood. The analysis of the reversal mechanisms is complicated not only by the fact that efflux transporters interact with structurally diverse compounds but also by the fact that substrates, modulators and inhibitors of P-gp and related transporters are bound from the lipid membrane and not from the aqueous phase. The aim of this review is to contribute to the understanding of MDR reversal by means of a physical chemical characterization of modulators and inhibitors. To this purpose the thermodynamic and kinetic aspects of drug-membrane and drug-transporter interactions are discussed. On this background it is possible to analyze the different mechanisms of modulation and inhibition of P-gp.

THERMODYNAMIC AND KINETIC ASPECTS OF DRUG-TRANSPORTER INTERACTIONS

P-gp-ATPase Activation and the Apparent Affinity to the Transporter. Drug-induced P-gp-ATPase activation is the basic process to be analyzed for an understanding of modulation and inhibition of P-gp. Drug-induced P-gp activation has been measured in inside-out cellular vesicles of MDR1 transfected cells either by monitoring the rate of inorganic phosphate release [41, 46, 55] or the rate of NADH production [56] as a function of drug concentration. In living, MDR1-transfected cells P-gp-ATPase activation has been measured by monitoring the *E*xtra*C*ellular *A*cidification *R*ate, ECAR [47]. Transporter activation leads to an increase in cellular metabolism, which can be monitored as increase in the extracellular acidification rate with a Cytosensor® Microphysiometer [57]. The rate of phosphate release and the ECAR show the same bell-shaped dependence on the logarithm of drug concentration, with an increase above basal values at intermediate concentrations and a decrease at high concentrations, respectively (Fig. **1**). The fact that most drugs first enhance and then reduce basal activation of P-gp depending on the concentration applied, bears interesting aspects for P-gp modulation. It may also explain

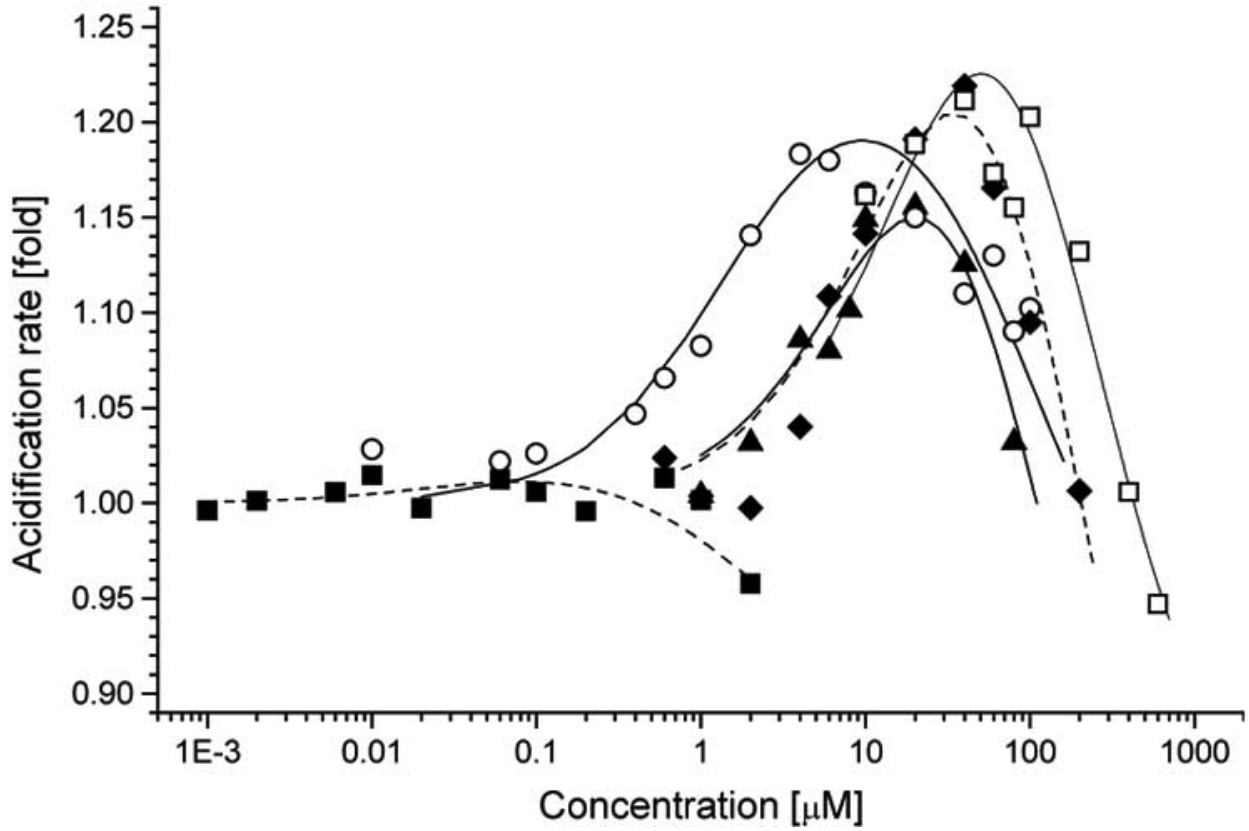
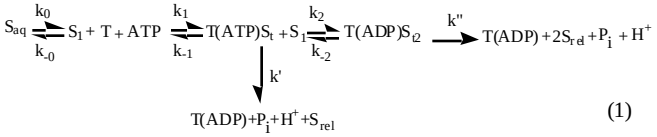


Fig. (1). P-gp-ATPase activation profiles obtained by measurements of extracellular acidification rates in living cells by means of a Cytosensor® Microphysiometer: Cyclosporin A (■), diltiazem ( ), progesterone (♦), trifluoperazine (s) and verapamil (O) in LLC-MDR1 cells. The solid or dashed lines correspond to modified Michaelis-Menten kinetics assuming activation with one and inhibition with two molecules bound according to eq. (2). Symbols represent an average value of n = 3-5 parallel measurements made with one single cell preparation. Adapted from [47].

inconsistencies in the classification of drugs with respect to their modifying action.

From thermodynamic and kinetic points of view substrates, modulators, and inhibitors can be treated alike. In the following we will therefore only discuss substrates if not otherwise required.

The rate of phosphate release [46] and the rate of extracellular acidification [47] have been analyzed quantitatively by means of a modified Michaelis-Menten equation, assuming first activation when only one substrate molecule, S_t, is bound, and inhibition when two substrate molecules, S_{t2}, are bound to the transporter, T. This is described in the following scheme



(1)

where S_{aq} and S_l are the substrates in the aqueous and the lipid phase, respectively, $T(ATP)S_t$ and $T(ATP)S_{t2}$ are transporter-ATP complexes with one, S_t, and two substrate molecules, S_{t2}, bound, respectively, T(ADP) is the transporter-ADP complex, P_i the inorganic phosphate, H^+, the proton released upon ATP hydrolysis, and S_{rel}, the substrate molecule released extracellularly. The parameters k_1, k_{-1}, k_2, and k_{-2} are the rate constants of the first and the second substrate binding step, and k' and k" the rate constants of the catalytic steps. Based on the above mechanism the rate equation for ATP hydrolysis can be written as

$$V_{Saq} = \frac{K_1K_2V_0 + K_2V_1C_{Saq} + V_2C_{Saq}^2}{K_1K_2 + K_2C_{Saq} + C_{Saq}^2}, \qquad (2)$$

where V_{Saq} is the rate of P_i release or of extracellular acidification as a function of the substrate concentration in solution, C_{Saq}. V_0 is the basal activity of P-gp-ATPase in the absence of substrate, V_1 is the maximum transporter activity (if only activation occurred) and V_2 is the minimum activity at infinite substrate concentration, K_1 is the dissociation constant of the first substrate binding site. At a substrate concentration $C_{Saq} = K_1$, half-maximum binding of the first binding site is reached. K_2 is the dissociation constant of the second substrate binding site. At a substrate concentration $C_{Saq} = K_2$, half-maximum binding of the second binding site is reached.

Under steady state conditions the inverse of the concentrations of half-maximum ($1/K_1$) and half-minimum activation ($1/K_2$) correspond to the affinities, K_{tw} and K_{tw2} of the substrate from water to the first and the second binding site of the transporter, respectively ($1/K_1 \sim K_{tw}$ and $1/K_2 \sim K_{tw2}$), provided the catalytic steps are slow. At low drug concentrations C_{Saq} eq. (**2**) simplifies to a Michaelis-Menten equation where K_1 corresponds to K_m, the Michaelis-Menten constant, and $1/K_m$ corresponds to the binding constant of the substrate from water to the transporter, K_{tw}.

Since P-gp and related transporters bind their substrates when they are inserted into the lipid phase, the transporter-binding step is preceded by a lipid-partitioning step as shown in scheme (1), i.e. first step on the left. The lipid-water partition coefficient is given by $(k_0/k_{-0}) = K_{lw}$ (for units cf. Table **1**). The binding constant of the substrate from water to the transporter $K_{tw} = (1/K_1)$ can thus be considered as the product of the lipid-water partition coefficient, K_{lw}, of the substrate and its binding constant from lipid to the transporter, K_{tl}

$$K_{tw} = K_{lw} \quad K_{tl}. \qquad (3)$$

The free energy of substrate binding from water to the transporter, ΔG_{tw}, can in turn be considered as sum of the free energy of substrate partitioning from water into the lipid membrane, ΔG_{lw}, and the free energy of substrate binding from lipid to the transporter, ΔG_{tl}

$$\Delta G_{tw} = \Delta G_{lw} + \Delta G_{tl}. \qquad (4)$$

The free energy of substrate binding from water to the transporter, ΔG_{tw}, (or apparent affinity to the transporter) is defined as

$$\Delta G_{tw} = - RT\ln C_w K_{tw} \quad RTC_w \ln K_1, \qquad (5)$$

where C_w is the concentration of water (55.5 mol/L at 25 °C) [58] (cf. Table. **1**). The free energy of substrate binding from water to the lipid membrane is defined as

$$\Delta G_{lw} = -RT\ln C_w K_{lw}. \qquad (6)$$

In contrast to previous assumptions [59, 60], the free energy of substrate binding from lipid to the transporter, ΔG_{tl}, cannot be measured directly, since membrane partitioning and receptor binding are tightly coupled processes and membrane partitioning cannot be saturated. However, the free energy of substrate binding from the lipid phase to the transporter, ΔG_{tl} can be estimated from ΔG_{tw} (eq. (4)) if the lipid - water partition coefficient, $K_{lw,}$ and

Table 1. Units of Partition Coefficients

Membrane or membrane mimicking system	*Binding constant or partition coefficient*	C_{Sm}	C_{Saq}
Lipid vesicles	K_{lw} [M^{-1}]	[mol/mol]	[mol/liter]
Lipid vesicles	γ_{lw} [dimensionless]	[mol/volume]	[mol/volume]
Lipid vesicles	K [dimensionless]	[mol/mol]	[mol/mol]
Hexane - water	γ_{hw} [dimensionless]	[mol/volume]	[mol/volume]
Octanol - water	P_{ow} [dimensionless]	[mol/volume]	[mol/volume]
Air - water interface	K_{aw} [M^{-1}]	-	[mol/liter]

ΔG_{lw} (eq. (6)) can be determined by an independent measurement.

Estimation of the Free Energy of Membrane Partitioning, ΔG_{lw}. Lipid-water partition coefficients, K_{lw}, cannot be measured in intact cells and therefore, appropriate membrane-mimicking or membrane model systems, respectively, are used. Best known in pharmaceutical sciences is the octanol-water partition coefficient [61]. The dielectric constant of octanol (10) is intermediate between that of the polar head group region (30) and that of the hydrophobic core region (2) of lipid bilayers. Hydrocarbons such as hexane and hexadecane have also been used (2) to measure partition coefficient. Hexane and octanol are, however, isotropic solvents; the lipid membrane, on the other hand, is an anisotropic liquid crystal with a well-defined short-range order. Compared to an organic solvent, model systems that induce anisotropic ordering of drug molecules are hence better suited to reflect the situation in a real membrane. Such model systems are either pure lipid bilayers or, perhaps surprisingly, the air-water interface. The latter forces amphiphilic molecules into an orientation in which the polar head groups are in the water phase, while the non-polar groups are aligned parallel in a medium of low polarity (air with 1).

Since all substrates and modulators and all membrane partitioning inhibitors of P-gp are surface-active compounds the air-water interface provides an ideal membrane mimicking system (for details cf. appendix).

Figure **2** shows the surface pressure, π, as a function of verapamil concentration (π-log C plot). From such a curve the air-water partition coefficient K_{aw}, and the cross-sectional area, A_D, of the molecule can be determined. Knowledge of the two parameters allows estimation of the lipid-water partition coefficient, K_{lw} according to [62]

$$K_{lw} = K_{aw} \cdot e^{-\pi_M A_D / kT}, \quad (7)$$

where π_M is the lateral packing density of the membrane. The basic difference between the air-water and the lipid-water interface is the packing density of the molecules involved. At the air-water interface the drug molecules aggregate spontaneously according to the concentration in solution. At the lipid-water interface the drug molecules penetrate between an already ordered layer of lipid molecules, which requires the energy $\pi_M A_D/kT$ [63]. The advantage of this approach is that it allows an estimate of the lipid-water partition coefficient for any membrane packing density. The latter can differ between different types of membranes.

The lateral packing density was determined as $\pi_M = 32 \pm 1$ mN/m for planar bilayers formed by 1-palmitoy-2-oleoyl-*sn*-3- phosphatidylcholine, POPC [64], which is the most abundant lipid in natural membranes, as $\pi_M = 30 \pm 1$ mN/m for bilayers of dimyristoyl phosphatidylcholine, DMPC [65], and as $\pi_M = 35 \pm 2$ mN/m for erythrocyte membranes [66]. High expression levels of P-gp were suggested to correlate with elevated levels of cholesterol and glycosphingolipids [67], which both increase the membrane

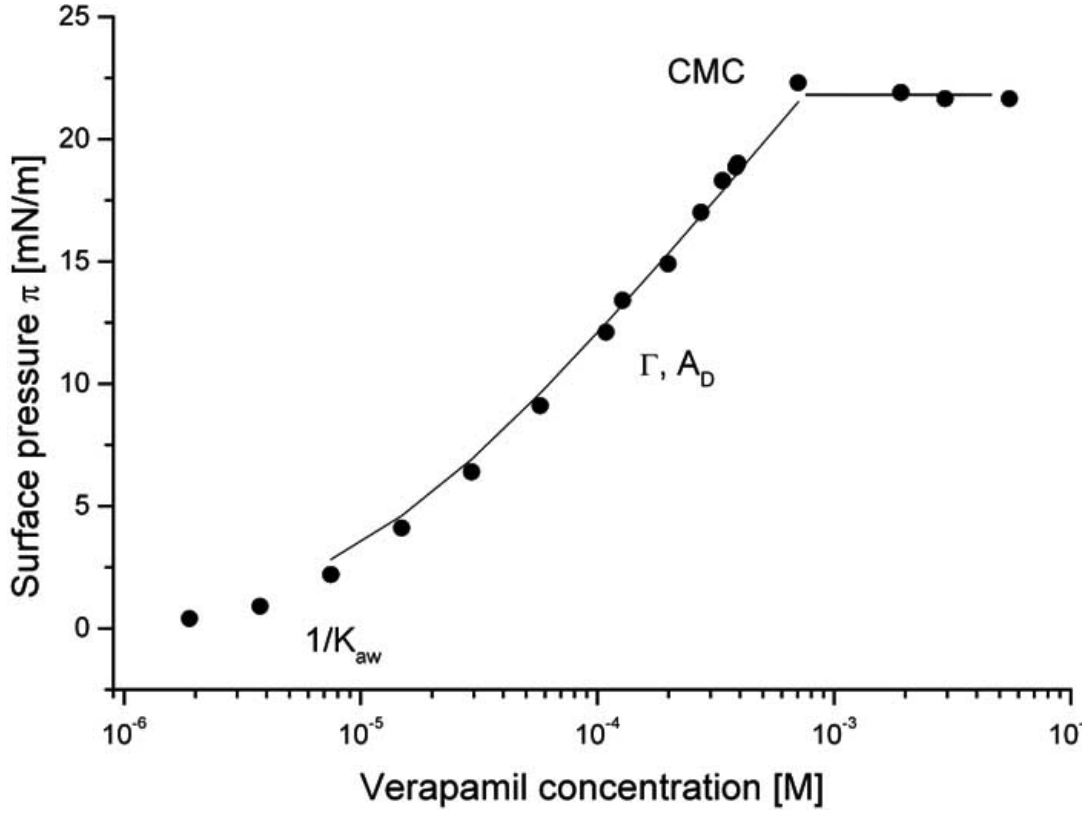


Fig. (2). Surface pressure as a function of verapamil concentration measured in 50 mM Tris/HCl at pH 8.0 (containing 114 mM NaCl) (π-log C plot) at room temperature. The solid line is the fit to the Szyszkowski equation, the air-water partition coefficient is $K_{aw} = 1 \cdot 10^5$ M^{-1}. The π-log C plot moreover yields the cross-sectional area of the molecule, A_D, the critical micelle concentration, CMC, and at high concentrations the solubility limit (not shown).

packing density. In the following we consider a membrane packing density, π_M = 30 mN/m as lower and π_M = 40 mN/m as upper limit.

Estimation of the Free Energy of Binding from Lipid to the Transporter, ΔG_{tl}. The free energy of substrate binding from water to P-gp has been determined as ΔG_{tw} ~ - 27 to - 56 kJ/mole using K_1 values published previously [47], the free energy of membrane partitioning was determined from surface activity measurements [68] using a membrane packing density of 30 mN/m as ΔG_{lw} ~ - 16 to - 39 kJ/mole, and the free energy of binding to the transporter as ΔG_{tl} ~ - 11 to - 17 kJ/mole [15]. The free energy of membrane partitioning, ΔG_{lw}, is generally more negative than the free energy of binding to the transporter, ΔG_{tl}. Therefore the membrane partitioning appears to be the most important step in the overall process. Only for very hydrophilic but efficient substrates the two free energies are in a similar range ($\Delta G_{lw} \sim \Delta G_{tl}$). If either ΔG_{lw} or ΔG_{tl} approach zero a compound will not interact with P-gp.

The present analysis suggests that K_m (should increase with increasing packing density of the membrane. This has indeed been demonstrated by functionally reconstituting P-gp in lipid bilayers of different composition [69].

Substrate-Transporter Interactions Based on H-bond Formation? The nature of the interaction between a substrate and its transporter is strongly determined by the dielectric constant of the local environment in which the molecular encounter takes place [15]. In a lipid environment van der Waals interactions are less pronounced than in an aqueous environment, while electrostatic or dipolar interactions are enhanced up to forty-fold (according to Coulomb's law) due to the low dielectric constant, ε 2, of the hydrophobic membranes core region. As a result, weak electrostatic interactions e.g. between the π-electrons of an aromatic ring and a cation [70] or H-bond interactions, which can be considered as dipole-dipole interactions may come into play. The relevance of H-bond formation for substrate recognition and binding is supported by the finding of specific H-bond acceptor patterns (called type I and type II units) (cf. Figure legend **3**) [32] in substrates and a high density of H-bond donor groups in the transmembrane sequences of P-gp [71].

The free energy of substrate binding from the lipid bilayer to the transporter P-gp, ΔG_{tl}, was therefore suggested to be the sum of the free energies resulting from H-bond formation between H-bond acceptor groups in the drug and H-bond donor groups in the transmembrane sequences of the transporter,

$$\Delta G_{tl} \approx \sum_{1} \Delta G_{Hî}. \qquad (8)$$

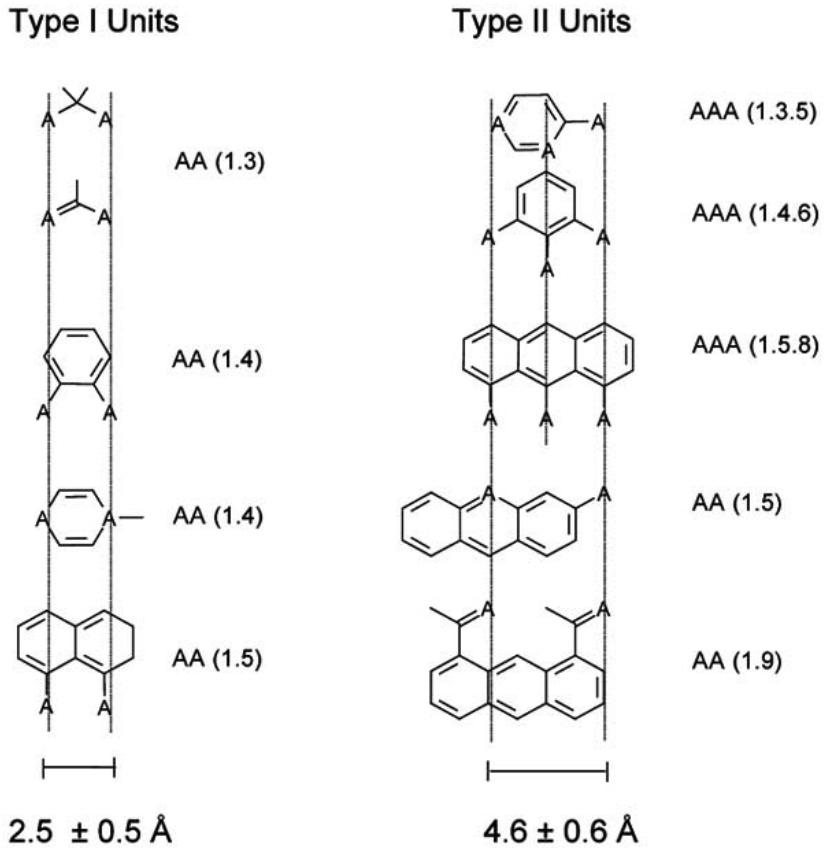


Fig. (3). H-bond acceptor patterns observed in P-glycoprotein substrates. Type I units: patterns formed by electron donor pairs with a spatial separation of 2.5 ± 0.3 Å. Type II units: patterns formed either by three electron donor groups with a spatial separation of the outer two electron donor groups of 4.6 ± 0.6 Å, or by two electron donor groups with a spatial separation of 4.6 ± 0.6 Å. *A* denotes a H-bonding acceptor group (electron donor group) and the numbers in brackets indicate the first and the *n*th atom with a free electron pair. Type I units are present in all substrates and type II units are present in all inducers of P-gp overexpression and in many substrates of P-gp. Adapted from [32].

To test this assumption we divided the measured ΔG_{tl} by the number of the relevant H-bond acceptor groups of a given substrate and obtained an average free energy per H-bond of ΔG_{Hi} = -5 to -10 kJ/mole (Gatlik-Landwojtowicz, Seelig, in preparation). Since the number of H-bonds formed simultaneously may have been overestimated, this result must be considered as a lower limit. It is consistent with values quoted in the literature for weak H-bonds [72].

In contrast to classical approaches based on one single "pharmacophore", the present approach based on incremental contributions of recognition sites is consistent with the different binding affinities of substrates to the transporter (for review see [73]).

Substrate-Transporter Interactions Determine the Rate of ATP Hydrolysis. It is reasonable to assume that the strength of drug-transporter interaction should influence the rate of ATPase activation. To further test the H-bond hypothesis we therefore plotted (Fig. **4**) the extracellular acidification rates V_1 and V_2 measured in MDR1 transfected LLC cells as a function of the estimated total free energy of H-bond formation between the substrates and the transporter (given in arbitrary H-bond units [EU_H]) [15]. For small substrates with a low affinity to the transporter (low value of arbitrary H-bonding units [EU_H] corresponding to low ΔG_{tl}) the maximum rate, V_1, is high and the minimum rate, V_2 is low. The large difference between V_1 and V_2 is typical for substrates, which show allosteric inhibition. As shown previously [47] these effects are independent of K_1 values. With increasing affinity to the transporter and increasing molecular size V_1 decreases and V_2 increases in an exponential manner. We will use this plot below to classify the substrates according to their free energy of interaction with the transporter.

Surface Activity Measurements Provide an Estimate of ΔG_{tw} (or K_1). The surface activity was measured for a number of substrates at pH 8. This pH was chosen to account for the pK_a shift of drugs upon insertion into the lipid membrane [74]. A plot of the logarithm of the inverse of the concentration of half-maximum P-gp activation ($1/K_m$

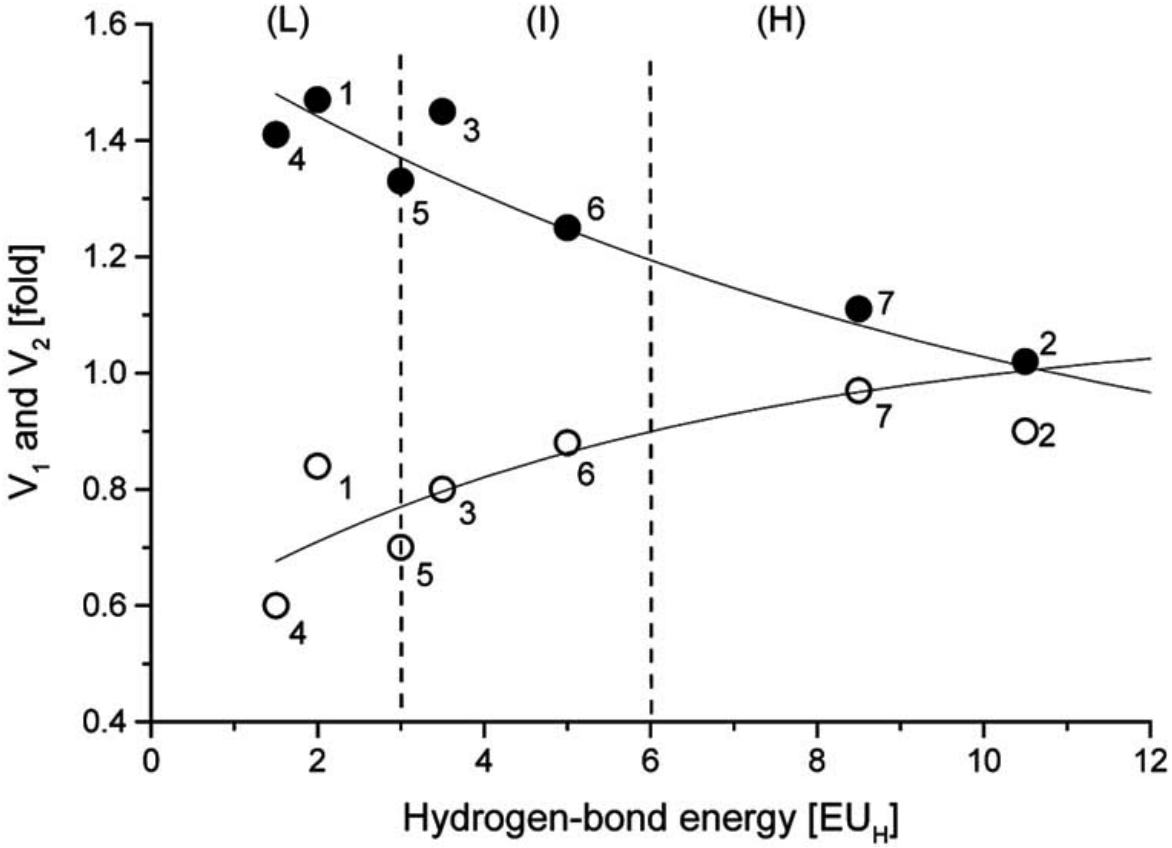


Fig. (4). Influence of the H-bonding energy per substrate on the rate of ATPase activation as determined by measurement of the extracellular acidification rate of intact MDR-transfected pig kidney cells [47]. The maximum, V_1, (minimum, V_2) rate of extracellular acidification given as fold of the basal extracellular acidification rate is plotted as a function of the H-bonding energy for amitriptyline (1), cyclosporin A (2), diltiazem (3), progesterone (4), trifluoperazine (5), verapamil (6), and vinblastine (7). It is assumed that the interaction between substrates and the transporter, ΔG_{tl}, within the lipid membrane is due to H-bond interactions ($\Delta G_{tl} \approx \sum_1 \Delta G_{Hi}$). Oxygen containing H-bond acceptors (>C=O, -OR) were assumed to contribute one H-bonding energy unit (EU_H = 1). Nitrogen has a similar H-bonding strength as oxygen. However, since it often appears in close proximity to oxygen (e.g. cyclosporin A) which reduces its electron donor strength, half the H- bonding energy value (EU_H = 0.5) was attributed to nitrogen. For simplicity the same value was also attributed to sulfur- and fluorine-containing groups (-SR, -CF) as well as to the phenyl group ($-C_6H_5$), although the electron donor strength of the latter two may be overestimated. The solid lines are exponential curves to guide the eye. (L), (I), and (H) represent classes of compounds with a low, an intermediate and a high binding affinity to the transporter, respectively. The classification is arbitrary.

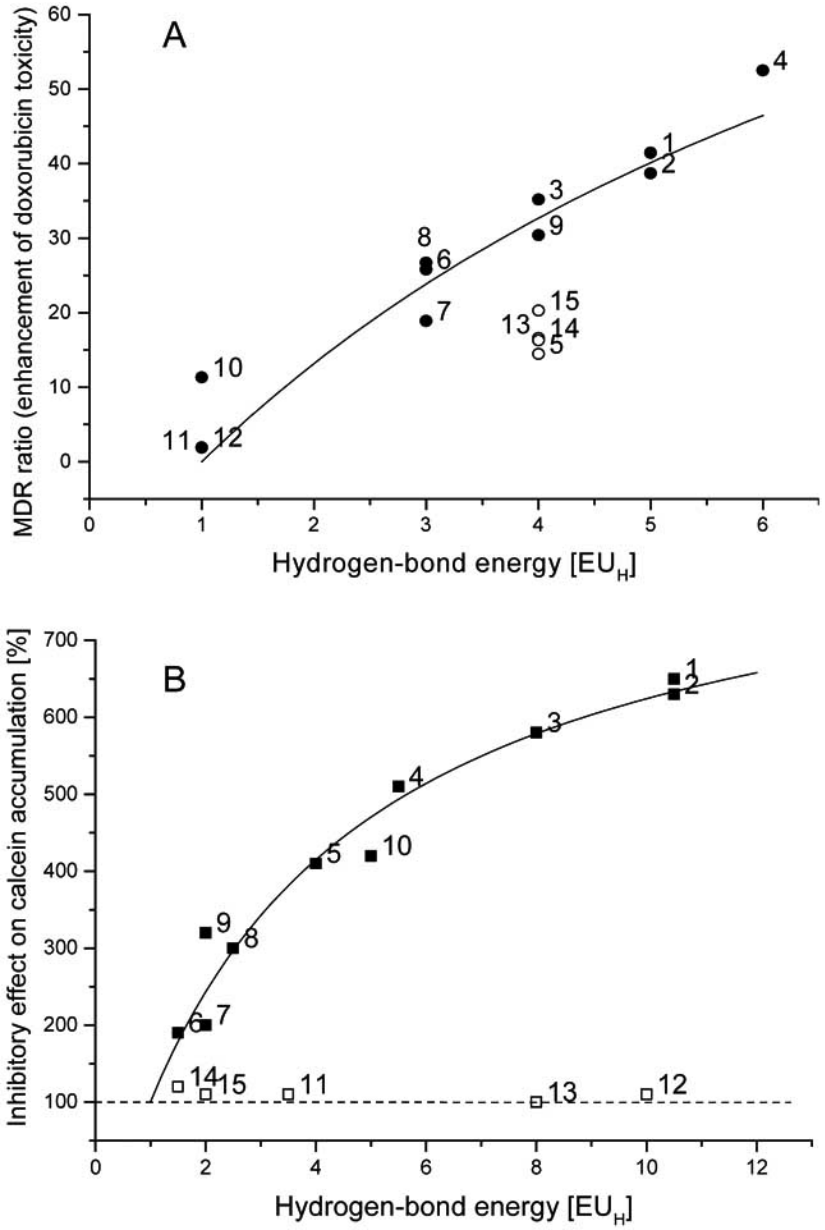


Fig. (5). A: Enhancement of doxorubicin cytotoxicity in LoVo-resistant cells by verapamil and analogues adapted from Toffoli *et al*. [76]. The results are expressed as fold increase in cytotoxicity represented by the ratio of doxorubicin IC_{50} in the absence and presence of verapamil and analogues. The verapamil concentrations used were the minimal cytotoxic concentrations (IC_{20}). The compounds are: verapamil (1), R-verapamil (2), nor-verapamil (3), gallopamil (4), LU46605 (5), devapamil (6), LU46324 (7), LU43918 (8), LU49667 (9), emopamil (10), S-anipamil (11), R-anipamil (12), LU49940 (13), LU48895 (14), LU51903 (15). The inhibitory potency of verapamil and analogues as predicted on the basis of the potential H-bonding energy given in arbitrary energy units, EU_H. Adapted from [68]. **B**: Inhibition of calcein-AM export (monitored by calcein formation) in isolated porcine brain capillary endothelial cells after incubation of the cells with 5 mM of P-gp modulators (adapted from [75, 86]) plotted versus H-bonding energy (EU_H). Compounds are: cyclosporin A (1), PSC 833 (2), ivermectin (3), ritonavir (4), nicardipine (5), saquinavir (6), morphine (7), loperamide (8), chinidine (9), verapamil (10), cortisol (11), erythromycin (12), digoxin (13), clozapine (14), yohimbin (15). The solid lines are fits to a saturation isotherm. Open symbols represent compounds which have a reduced tendency to partition into the lipid membrane and were therefore not included in the fits, e.g. compound (5) which carries two cationic groups and compounds (13), (14), (15) which most likely form micelles in panel A; and compounds (11), (12) and (13) which exhibit low air-water partition coefficients, K_{aw}, and/or a large cross-sectional areas, A_D, (cf. eq. (7)) in panel B.

$\sim K_{tw}$) (which is proportional to ΔG_{tw}) as a function of logarithm of the air-water partition coefficients, K_{aw} (which is proportional to ΔG_{aw}) reveals a linear correlation ($m = 0.94 \pm 0.09$, $R = 0.96$) of the form [68]

$$K_{aw} \cdot K_m \approx 1$$

which is equivalent to

$$\Delta G_{aw} \quad \Delta G_{tw}. \qquad (9)$$

Rewriting eq. (7) in a logarithmic form and combining it with eq. (6) shows that the free energy of partitioning into the lipid-water interface, ΔG_{lw}, corresponds to the free energy of partitioning into the air-water interface, ΔG_{aw}, plus the additional work to penetrate into the bilayer

$$\Delta G_{lw} = \Delta G_{aw} + \pi_M N_A A_D \quad (10)$$

Combining the experimental result given in eq. (9) with equation (4) and (10) leads to the following approximation

$$\Delta G_{tw} \quad \Delta G_{aw} = \Delta G_{lw} - \pi_M N_A A_D \quad (11)$$

or

$$\Delta G_{tl} \quad - \pi_M N_A A_D. \quad (12)$$

Eq. (12) suggests a correlation between the cross-sectional area, A_D, of the compound and the free energy of binding to the transporter, ΔG_{tl}. A correlation between molecular size and the rate of ATP hydrolysis has been observed previously [45]. However, it seems likely that not the size as such but rather the concomitant increase in the probability to find interactive groups determines the substrate-transporter interaction within the membrane. For the substrates investigated this is indeed supported by the finding of a direct correlation between the free energy of H-bond formation given in arbitrary units [EU_H] and the cross-sectional area, A_D.

Competition Assays. The inhibition constant K_i, is inversely related to the binding affinity to the transporter. In the frame of the H-bond concept this would mean that the inhibition constant decreases with the potential of the compound to form H-bonds with the transporter. This effect is illustrated in (Fig. **5**), which displays two types of competition assays [15], a calcein-AM assay (A) [75] and a cytotoxicity assay (B) [76]. Figure **5A** and **B** show that the interaction of a drug with the transporter can be well predicted on the basis of H-bond interactions provided the membrane concentration of drugs is sufficiently high.

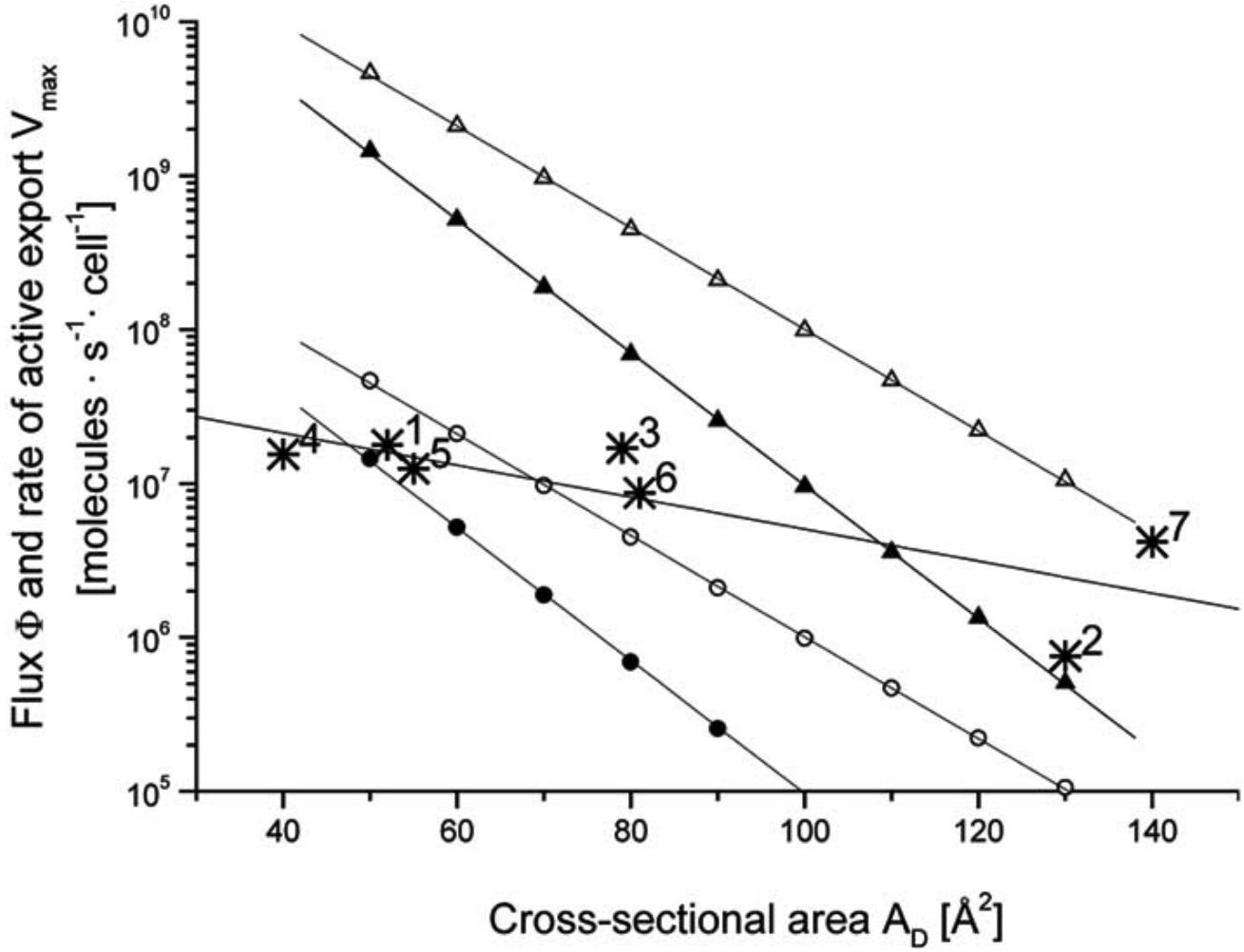
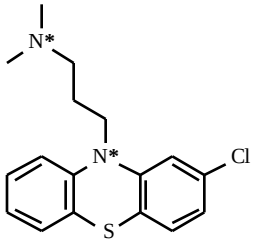


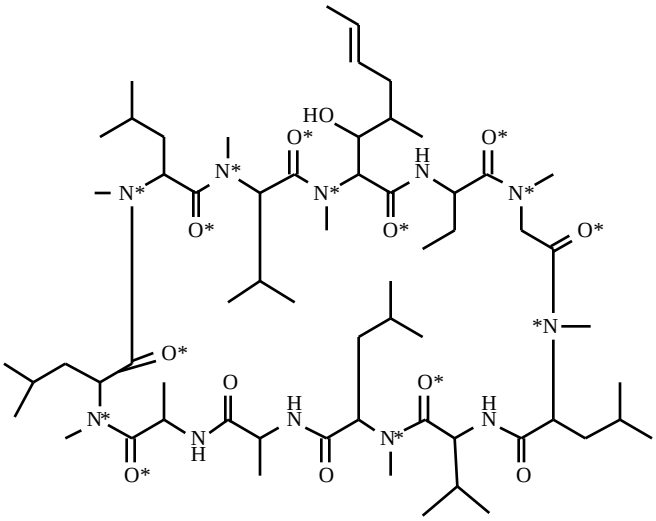
Fig. (6). Passive influx, Φ, and active efflux, $-V_1$, both given in [molecules·s^{-1}·$cell^{-1}$] are plotted as a function of the cross-sectional area, A_D. Influx is calculated for hypothetical non-electrolytes with increasing cross-sectional areas, A_D, an air-water-partition coefficient, $K_{aw} = 100\ M^{-1}$, two different drug concentrations, C = 0.01 μM (circles) and 1 μM (triangles) and two different packing densities $\pi_M = 30$ mN/m (open symbols) and $\pi_M = 40$ mN/m (solid symbols) (where the higher packing density most likely reflects that of cells with a high expression levels of P-gp). The viscosity was η = 1 poise, and the bilayer thickness Δx = 50 Å. The surface area of cells was chosen as $A_{cell} = 2.27 \cdot 10^{-7}$ cm^2, where 20 % of this area was assumed to be a pure lipid bilayer. The V_1 data given were obtained by means of a Cytosensor® in MDR1-transfected LLC cells [47]. Numbers correspond to those in Fig. (**4**). The maximum rate of effective transport out of the cell is assumed to correlate with ATPase activation.

A: First generation inhibitors

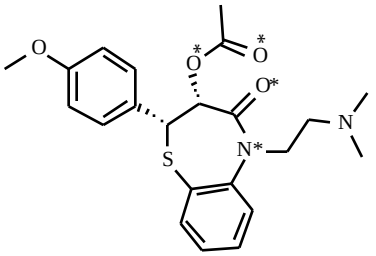
Chlorpromazine



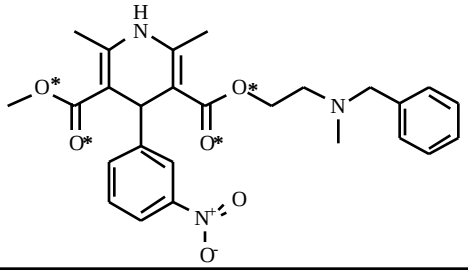
Cyclosporin A



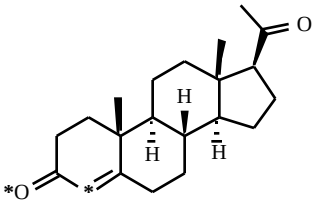
Diltiazem



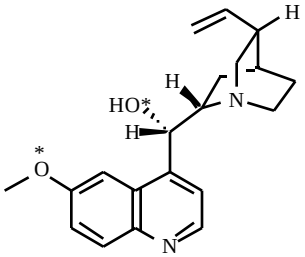
Nicardipine



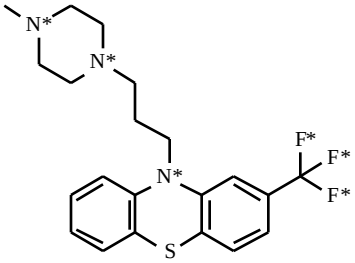
Progesterone



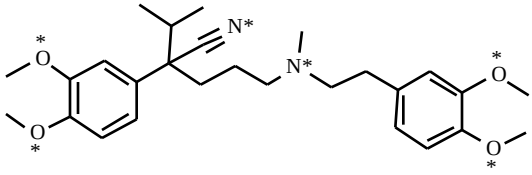
Quinidine



Trifluoperazine

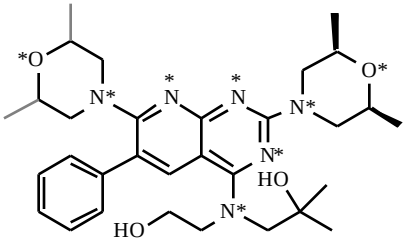


Verapamil +/-



B: Second generation inhibitors

BIBW22BS



Dexniguldipine

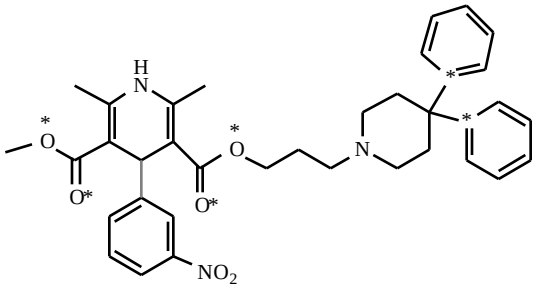
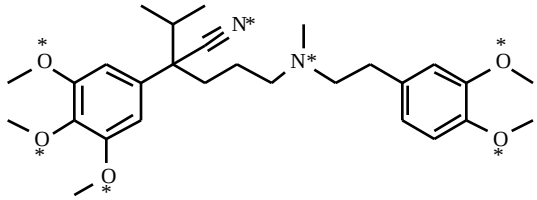


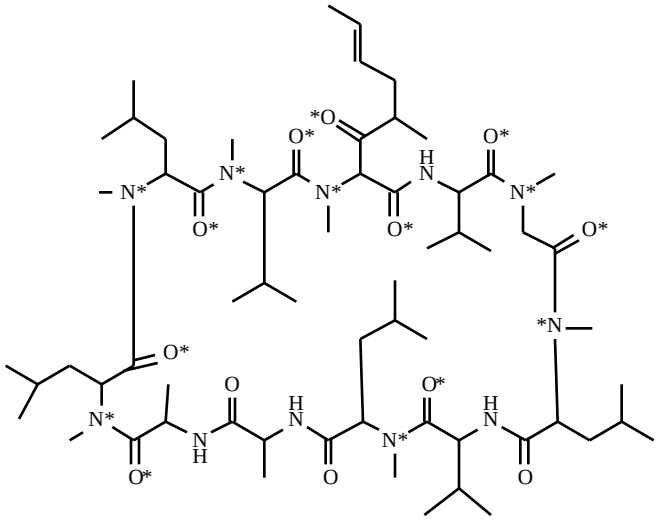
Fig. (7). contd.....

B: Second generation inhibitors

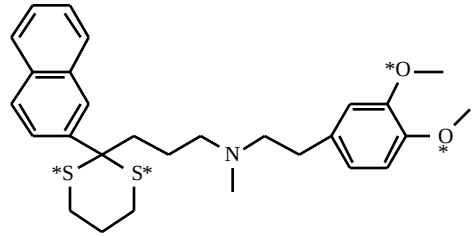
Gallopamil



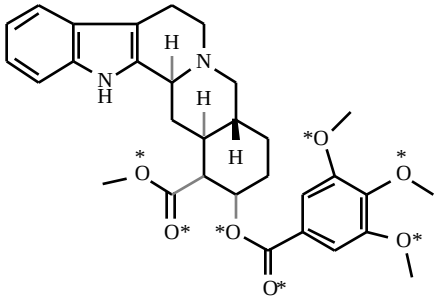
PSC833



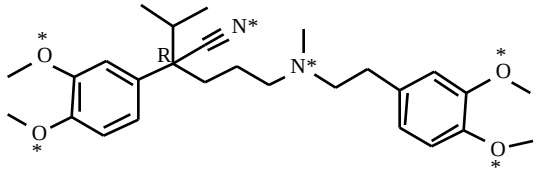
Ro11-2933



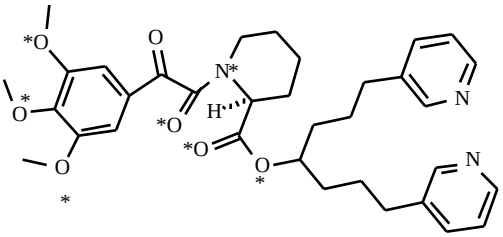
TMBY trimethoxybenzoylyohimbine



Verapamil (R)

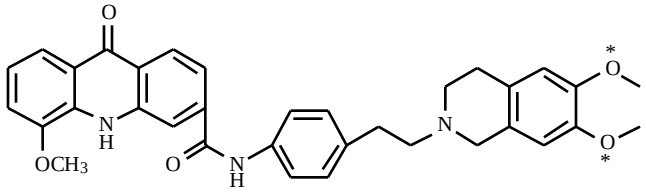


VX-710



C: Third generation inhibitors

GF120918



LY335979

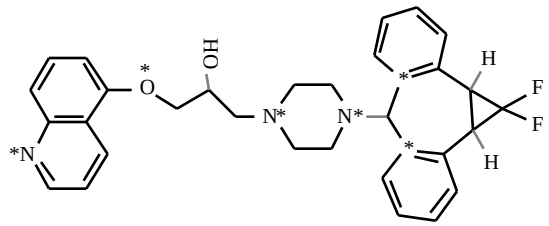


Fig. (7). contd.....

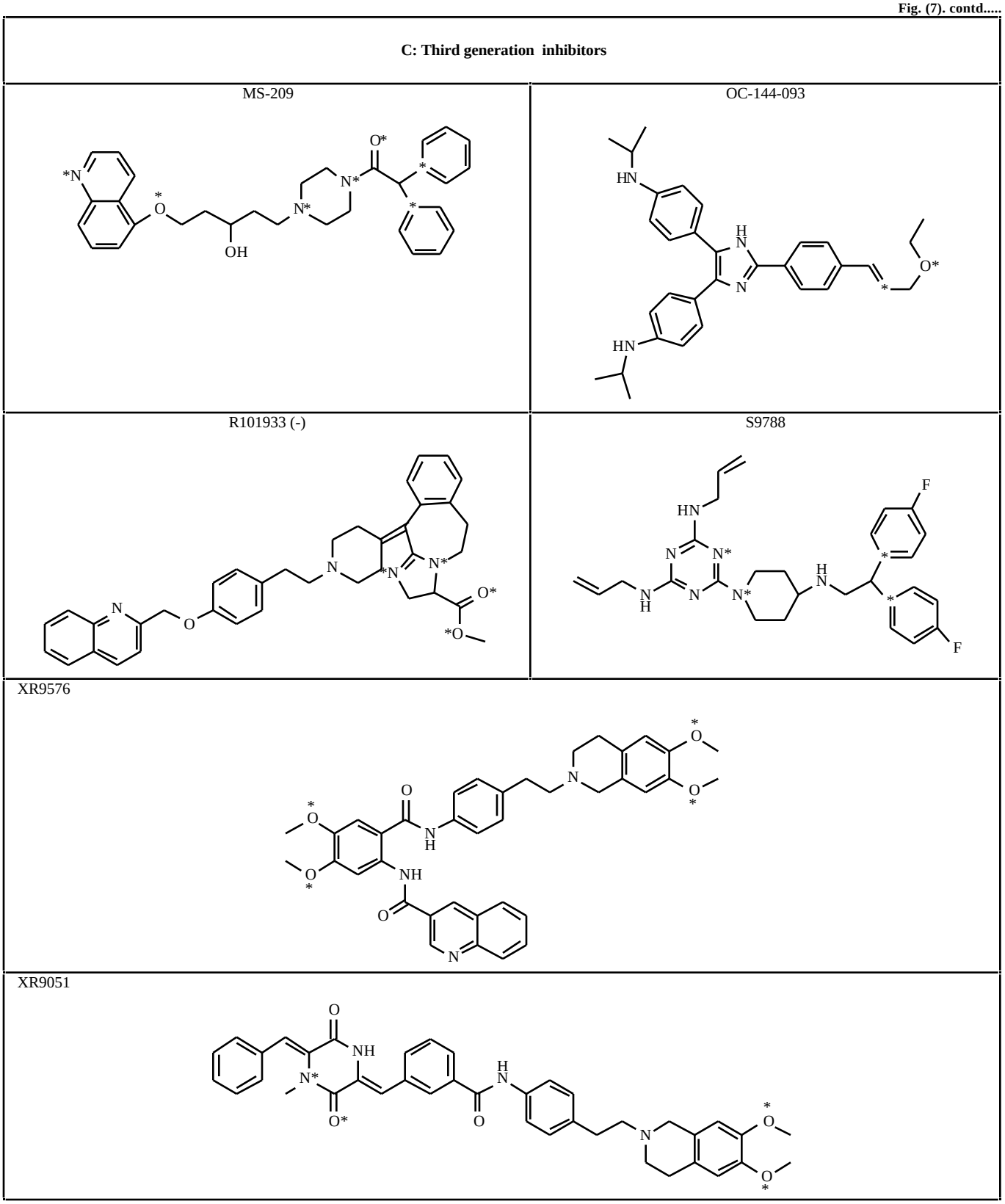


Fig. (7). Inhibitors and modulators of P-glycoprotein. The H-bond acceptors in possible type I and type II units are indicated by stars.

Net Transport by P-gp. Net transport out of the cell, J, is the sum of passive influx into the cell, Φ [77-79] and active efflux out of the cell, ($-V_{Saq}$), where the latter is related to the rate of ATP hydrolysis (eq. (2))

$$J = \Phi - V_{Saq}. \quad (13)$$

The influx, Φ, is given by the product of the permeability coefficient, P, and the gradient between the extracellular and the intracellular drug concentration, ΔC. If

only the initial phase of drug application is considered, the intracellular drug concentration is negligible, and the expression of the flux simplifies to

$$\Phi = C_{Saq} \cdot P, \quad (14)$$

where C_{Saq} is the extracellular aqueous drug concentration (details are discussed in Gatlik-Landwojtowicz, Seelig, in preparation).

Figure **6** displays a comparison of the passive influx, Φ, and active efflux, V_1 as a function of the molecular cross-sectional area, A_D. The data for active export, V_1, correspond to the data given in (Fig. **4**), which were measured for a series of compounds (electrolytes and non-electrolytes) in MDR1 transfected LLC cells [47]. The influx, Φ, was calculated for a hypothetical set of non-electrolytes with increasing cross-sectional areas, A_D, and a constant air-water partition coefficient, $K_{aw} = 10^2$ M^{-1} at two different membrane packing densities (π_M = 30 mN/m and π_M = 40 mN/m) and two different concentrations (C_{Saq} = 0.01μM and 1μM). The membrane thickness and the viscosity were chosen as Δx = 50 Å, and, η = 1 poise, respectively.

For molecules with small cross-sectional areas ($A_D \sim 50$ Å^2) the rate of passive influx, Φ, is orders of magnitude higher than the rate of active efflux, V_1, (at least at high concentrations) and therefore no net transport is observed despite the fact that ATPase activation and thus effective transport is high. In contrast, for molecules with large cross-sectional areas ($A_D > 80$ Å^2) the rate of passive influx, Φ is lower than the rate of active efflux, V_1, and as a consequence, net transport is observed. Figure **6** moreover demonstrates that the influx, Φ, decreases with increasing membrane packing density, π_M, which leads to a concomitant increase in net transport. It is interesting to note that high expression levels of P-gp seem to correlate with elevated levels of cholesterol and glycosphingolipids [67], which both increase the membrane packing density. This suggests a synergistic role of the lipid bilayer for net transport by P-gp.

MODULATORS AND INHIBITORS

A representative selection of first-, second- and third-generation modulators and inhibitors is shown in (Fig. **7**) and (Table **2**). Their interaction with P-gp will be discussed in the frame of the above discussion. The compounds were arbitrarily divided in three classes with a low ($EU_H \leq 3$) (class (L)), an intermediate ($3 < EU_H < 6$) (class (I)), and a high potential to form H-bonds with the transporter, ΔG_{tl} ($EU_H \geq 6$) (class (H)) (cf. Fig. **4**). To compensate for the fact that K_{aw} and A_D has not yet been measured for all inhibitors the number of atoms in the extended main chain of the molecule (column 8) and octanol-water partition coefficients (log P values) (column 10) are given in Table **2**. Columns 11-14 summarize drug interactions with proteins, such as P-gp-ATPase activation, the interaction with MRP1, the interaction with cytochrome P450 3A4, and pharmacokinetic interactions with other drugs.

First-Generation Modulators. First-generation modulators comprise compounds of all three classes (L, I, H) (cf. Table **2**). Compounds of class (L) significantly reduce the rate of ATPase activation if applied at high concentrations (low V_2 values) as seen in (Fig. **4**). In terms of a two-site binding model [46, 47] inhibition or modulation arises if a second allosteric binding site is occupied. These molecules have a low affinity to the transporter. Since they are small they are able to occupy the two discrete binding sites. Due to their rather small cross-sectional areas ($A_D < 60$ Å^2) the influx, Φ, can be orders of magnitude higher than the rate of active efflux, V, especially, if compounds are applied at the high concentrations required for inhibition (Fig. **5**). The compounds were originally developed for other therapeutic indications than P-gp and therefore the high concentrations required for modulation or inhibition can be toxic [80].

Class (I) comprises compounds such as verapamil. These compounds also reduce the rate of activation at high concentrations, however, to a lesser extent than compounds of class (L) (cf. Fig. **4**). However, they exhibit higher affinities to the transporter due to the higher number of H-bond acceptor groups (Fig. **5**) and in addition higher lipid-water partition coefficients, K_{lw}, the concentrations of half-maximum activation/inhibition, $K_{1,2}$ are therefore lower and the compounds are thus more potent. Moreover, they exhibit larger cross-sectional areas, A_D, and therefore efflux, Φ, is somewhat smaller. Nevertheless, verapamil still shows toxic side effects at the high concentrations required for inhibition of P-gp.

Class (H) comprises even larger compounds ($A_D > 100$ Å^2) with a higher number of H-bond acceptor patterns. Compounds with this high potential to form H-bonds (e.g. cyclosporin A) can bind efficiently to the transporter. Most likely they occupy a large proportion of the binding sites in the transporter and therefore act as competitive inhibitors (cf. Fig. **3** in [68], data adapted from [41]). They slow down the rate of P-gp ATPase activation even at low concentrations. Since these compounds have large cross-sectional areas, A_D, influx is low (Fig. **6**) and P-gp can cope with influx to keep the drug out of cells.

The drawback of compounds of class (H), and to some extent also of those of class (I), is their influence on the pharmacokinetics and biodistribution of anticancer drugs. Compounds with a higher number of type I and type II units not only have a higher affinity to P-gp but also to other transporters and to the metabolizing proteins like e.g. cytochrome P450 (and especially P450 3A) [49, 81] due to overlapping substrate specificities. A compound with a high affinity to these proteins thus prevents metabolism of the co-administered anticancer drugs and increases their systemic toxicity.

Second-Generation Modulators. Second-generation modulators are essentially stereoisomers or analogs of first-generation inhibitors e.g. verapamil and cyclosporin A. Since they have no other targets than P-gp they are more specific and less toxic. Like the parent compounds they belong to class (I) and (H). Their influence on the parmacokinetics and biodistribution of anticancer drugs lead to the discontinuation of their development.

Third-Generation Inhibitors. Third-generation inhibitors interact with P-gp by blocking the efflux of substrates *in vitro* as well as *in vivo*. Only little experimental data is yet available. With respect to H-bond acceptor patterns third-

Table 2. First-, Second- and Third-Generation Modulators and Inhibitors of P-Glycoprotein

1 Gene-ration	2 Class	3 Drug	4 Formula	5 MW Base	6 H-bond acceptor units	7 H-bond donor	8 Length of main-chain	9 pKa[1]	10 LogP$_1$	11 P-gp ATPase activity	12 MRP1 interaction	13 CYP 3A4 interaction	14 PK[2]	15 Clinical phase [87]	16 Ref.
1st	(L)	Chlorpromazine	$C_{17}H_{19}ClN_2S$	318.86	1	-	10	9.43	5.36	act	inh	sub	yes		[88, 89]
	(L)	Progesterone	$C_{21}H_{30}O_2$	314.46	1.5	-	12	-	4.04	act	n.d	sub	yes		[90-92]
	(L)	Trifluoperazine	$C_{21}H_{24}F_3N_3S$	407.50	3	-	14	7.82	5.11	act	inh	weak mod	yes		[93, 94]
	(I)	Diltiazem	$C_{22}H_{26}N_2O_4S$	414.52	3.5	-	14	8.91	4.53	act	n.d	inh	yes		[95, 96]
	(I)	Verapamil +/-	$C_{27}H_{38}N_2O_4$	454.60	5	-	17 folded	9.03	4.91	act	inh	sub, inh	yes		[97]
	(H)	Cyclosporin A	$C_{62}H_{111}N_{11}O_{12}$	1202.60	10.5	4xNH	24 cyclic	-	n.d	inh	inh	sub, inh	yes		[98]
2nd	(L)	Ro11-2933	$C_{28}H_{35}NO_2S_2$	481.72	3	-	18	9.14	6.43	n.d	n.d	n.d	no		[99]
	(I)	Verapamil (R)	$C_{27}H_{38}N_2O_4$	454.60	5	-	17 folded	9.03	4.91	act	n.d	sub	n.d	Discontinued	[100-102]
	(I)	BIBW22BS	$C_{30}H_{43}N_7O_4$	565.71	5	2xOH	13	8.89	1.40	n.d	n.d	n.d	n.d		[103]
	(I)	Dexniguldipine	$C_{36}H_{39}N_3O_6$	609.71	5	NH	19	8.53	n.d	n.d	n.d	n.d	n.d	Discontinued	[104, 105]
	(H)	Gallopamil	$C_{28}H_{40}N_2O_5$	484.64	6	-	17	9.01	4.61	n.d	n.d	no	n.d		[76, 106]
	(H)	VX-710 Biricodar	$C_{34}H_{41}N_3O_7$	603.71	6.5	-	20	5.88	4.08	act	inh, mod of BCRP	n.d	yes	Phase II	[107, 108]
	(H)	TMBY trimethoxy benzoylyohimbine	$C_{31}H_{36}N_2O_7$	548.63	7	NH	20	8.09	4.92	no effect	n.d	n.d	n.d		[80, 109]
	(H)	PSC 833 (Valspodar)	$C_{63}H_{111}N_{11}O_{12}$	1214.62	10.5	4xNH	24 cyclic	-	n.d	inh	weak inh	sub	yes	Phase III, discontinued	[110, 111]
	(L)	OC-144-093 (ONT-093)	$C_{32}H_{38}N_4O$	494.67	1	3xNH	20	5.89	8.71	n.d	no	no	no	Phase I	[112, 113]
3rd	(L)	S9788	$C_{28}H_{33}N_7F_2$	505.61	2	3xNH	19	9.37	3.24	n.d	mod	n.d	n.d		[114, 115]
	(L)	GF120918 (Elacridar)	$C_{34}H_{33}N_3O_5$	563.64	2	2xNH	24	7.83	6.91	inh	no, mod of BCRP	no	no	Phase I	[116, 117]
	(L)	R101933 (-) (Laniquidar)	$C_{37}H_{38}N_4O_3$	586.72	3	-	26	10.31	5.93	n.d	n.d	no	no		[118]
	(I)	XR9051	$C_{39}H_{38}N_4O_5$	642.74	3.5	2xNH	30	n.d	n.d	inh	n.d	n.d	n.d		[119]
	(I)	XR9576 (Tariquidar)	$C_{38}H_{38}N_4O_6$	646.73	4	2xNH	25	7.84	6.21	inh	no	n.d	no	Phase III	[120, 121]
	(I)	LY335979 Zosuquidar	$C_{32}H_{31}N_3O_2F_2$	527.60	4.5	OH	20	n.d	n.d	n.d	no	weak inh	no	Phase II	[122, 123]
	(I)	MS-209	$C_{32}H_{35}N_3O_3$	509.64	4.5	OH	22	n.d	n.d	n.d	inh	n.d	n.d		[124]

[1]from Scifinder, calculated using advanced chemistry development (ACD); [2]PK-pharmacokinetic interactions with other drugs, act - activation, inh - inhibiton (inhibitor), sub - substrate, mod-modulator.

generation inhibitors (GF120918, LY335979, MS-209, OC-144-093, R101933, S9788, XR9051, XR9576) belong to class (L) or at most to the low range of class (I). In contrast to compounds of class (L) and (I) from first- and second-generation inhibitors they seem in general to inhibit P-gp even at low concentrations. This suggests a high lipid-water partition coefficient, K_{lw}, which is supported by the comparatively high octanol-water partition coefficients (log P values).

A closer look at the structures of third-generation inhibitors reveals that compounds are distinctly longer than first-generation modulators with an average of 23 atoms in the extended main chain as compared to up to 14 atoms for first generation modulators. They are even longer than a typical lipid molecule such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, which exhibits 16 plus ~ 4 atoms (*sn*-1 fatty acyl chain plus ester linkage and glycerol backbone) in an extended conformation perpendicular to the bilayer surface taking into account that the *sn*-2 chain is bent close to the membrane-water interface and that the head group is oriented almost parallel to the bilayer surface [82]. The quotient of length of the main chain of the drug molecule when inserted in the lipid membrane, L_{DM}, and of the length the lipid molecule, L_L, is thus $(L_{DM}/L_L) > 1$. Moreover, the H-bond acceptor patterns are surprisingly far apart from each other (e.g. XR9051). In contrast to e.g. verapamil, which most likely adopts a folded, amphiphilic structure in the membrane, third-generation inhibitors often show a rigidified tertiary amino group and therefore most likely adopt an extended conformation. This may render transport or flip-flop more difficult. They further differ from first- and second-generation modulators in that they exhibit a distinctly higher number of H-bond donor groups (e.g. secondary amino or amide groups) (cf. Table **2**, column 7), which generally seem not to interact with P-gp [32]. Interestingly, a non-competitive interaction was observed for the third-generation inhibitor, XR9576 [83], which is in agreement with the low H-bonding affinity of the compound. Third-generation inhibitors appear to be well tolerated in combination with anticancer drugs that are P-gp substrates due to the lack of significant pharmacokinetic interactions [48], which is also consistent with the relatively low number of H-bond acceptor patterns.

Detergent-Like Modulators. Molecules such as polyethylene oxides [53, 84] or pentylglycerol ([52]; Gatlik-Landwojtowicz, Erdlenbruch, Seelig, in preparation) interact with P-gp most likely also due to their H-bond acceptor groups. The probability of type I unit formation e.g. in polyethylene oxides is, however, low due to the high flexibility of the molecule and therefore binding is less efficient than in rigid type I units (Nervi and Seelig, in preparation). Despite the high number of H-bond acceptor groups in certain detergent-like modulators they therefore rather belong to class (L) or (I). In addition to their interaction with P-gp they also decrease the packing density of membranes. This leads on one hand to an increase in the lipid-water partition coefficient, K_{lw}, and a concomitant decrease in the concentration of half-maximum activation,

Table 3. Modulators and Inhibitors are Divided into Three Classes Exhibiting a Low (L), an Intermediate (I), or a High (H) Potential to Interact with the Transporter

Physical-chemical parameters (A)	Physical-chemical parameters (B) influenced by parameters (A)	Class (L) *1st generation Modulators*	Class (L) *3rd generation Inhibitors*	Class (I) *1st, 2nd generation Modulators*	Class (H) *1st, 2nd generation Modulators*
ΔG_{tl} estimated		$EU_H \leq 3$	$EU_H \leq 3$	$3 < EU_H < 6$	$EU_H \geq 6$
ΔG_{tl} measured		low	low	intermediate	high
	V_1 (V_{max})	high	low / negligible	intermediate	low
	V_2 (V_{min})	low		intermediate	high
	Type of modulation, inhibition	allosteric, non-competitive	allosteric, non-competitive	allosteric or competitive	competitive
	Effect on Pharmacokinetics	low	low	intermediate	high
ΔG_{tw} measured ΔG_{lw} measured		low	high	intermediate	intermediate-high
	K_1 (K_m)	high	low	intermediate	low
	Potency	low	high	intermediate	high
A_D		small	n.d.	intermediate	large
	Flux, Φ into cell	high	n.d.	moderate	low
	Net transport	no or low	no or low	moderate	high
(L_{DM} / L_L)		< 1	> 1	< 1	< 1
	Transport or flip-flop	yes	no	yes	yes

K_1, [85] and on the other hand to an increase in influx, Φ (cf. Fig. **6**). The two factors synergistically enhance membrane permeation of drugs and thus reverse MDR. The thermodynamic, kinetic, and structural parameters and their effect on the properties of modulators and inhibitors are summarized in Table **3**.

CONCLUSIONS

Binding of a drug from water to the transporter occurs in two steps, a partitioning step from water to the lipid membrane, characterized by a lipid-water partition coefficient, K_{lw}, and a binding step from the lipid membrane to the transporter, characterized by a binding constant, K_{tl}. The binding constant, from water to the transporter, K_{tw}, can thus be expressed as product of two individual binding constants K_{lw} and K_{tl}, and the free energy of binding, ΔG_{tw}, as sum of two corresponding free energies, ΔG_{lw} and ΔG_{tl}. The free energies, ΔG_{tw} and ΔG_{lw} can be determined independently, which allows an estimate of the free energy of binding from lipid to the transporter, ΔG_{tl}. The value of ΔG_{tl} obtained is consistent with a model in which the drug interacts with the transporter primarily *via* H-bond formation in type I and type II units ($\Delta G_{tl} \approx \sum_i \Delta G_{Hi}$). The free energy of membrane partitioning, ΔG_{lw}, seems to be generally more negative than the free energy of transporter binding and thus determines the potency of the drug. The substrate-transporter interaction characterized by ΔG_{tl} determines in turn the rate of activation and the type of inhibition. Small values of ΔG_{tl} typically lead to allosteric, non-competitive interactions while large values lead to competitive interactions. Compounds, which show large negative ΔG_{tl} values for P-gp are expected to also show large negative ΔG_{tl} values for cytochrome P450, due to overlapping substrate specificities. This leads to pharmacokinetic drug-drug interactions.

Net transport, J, which is the sum of passive drug influx, Φ, and active drug efflux, $-V_{Saq}$ ($J = \Phi - V_{Saq}$) increases with increasing cross-sectional area, A_D, and increasing cationic charge (or pK_a) of the drug and the packing density of membrane, π_M. For transport an upper limit of the cross-sectional area, A_D, and of the pK_a value has not yet been observed and may be set only by the energy required for a compound to penetrate into the membrane and to reach the site of interaction. Since P-gp does not transport compounds with a negative charge a lower limit ~ pK_a5 is given. From all parameters determined, A_D (which is not necessarily identical with the molecular weight of the compound) and the charge (or pK_a) seem to be the parameters, which ultimately determine whether or not a compound will reach the cell.

Modulators of P-gp are at the same time substrates. Those which exhibit a small negative free energy of interaction with the transporter generally show allosteric inhibition, and those which exhibit a large negative free energy of interaction, competitive inhibition. If compounds exhibit at the same time small cross-sectional areas they reach the cytosol despite being transported. If they are large they do not reach the cytosol (net transport). *Inhibitors* bind to P-gp, without being transported. Strictly speaking this seems to be true only for third-generation inhibitors which seem unable to adopt a folded conformation and tend to be longer than an average lipid molecule. There may thus exist an upper length limit for transport by P-gp.

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APPENDIX

Air-Water Partition Coefficient and Cross-Sectional Area. After injection into an aqueous solution surface active compounds partition between the bulk and the air-water interface and thereby lower the surface tension of the aqueous solution, γ_0, to a value, γ. The difference, $\pi = \gamma_0 - \gamma$, is called the surface pressure and can be measured e.g. by means of a Wilhelmy plate in a monolayer trough. The interfacial adsorption as a function concentration is described by the Gibbs adsorption isotherm

$$d\gamma = -RT\Gamma d \ln C_{Saq} \qquad (15)$$

where RT is the thermal energy per mole, C_{Saq} the bulk concentration of the amphiphilic compound and Γ is the excess surface concentration. The latter can be written as

$$\Gamma = \frac{\Delta n}{A} = \frac{1}{N_A A_S} \qquad (16)$$

where Δn is the surface excess of molecules in the interface, A is the total area of the aqueous surface, N_A is the Avogadro constant and A_S is the surface area requirement of the compound at the air-water interface. In the absence of electrostatic repulsion (e.g. at pH 8 for weak bases) A_s corresponds to the cross-sectional area, A_D, of the molecule. A plot of π versus log C_{Saq} yields a straight line as long as Γ is constant. The surface adsorption process can also be described by a Langmuir adsorption isotherm

$$\Gamma = \Gamma_\infty \frac{K_{aw} C_{Saq}}{1 + K_{aw} C_{Saq}} . \qquad (17)$$

Here the excess surface concentration, Γ, is no longer constant but increases with increasing bulk concentration, C_{Saq}, up to a limiting value, Γ_∞, where K_{aw} is the air-water partition coefficient with the dimension [M^{-1}].

An integral form of the Gibbs adsorption isotherm combined with the Langmuir adsorption isotherm leads to the Szyszkowski equation

$$\pi = \Gamma_\infty RT \ln (1 + K_{aw} C_{saq}) . \qquad (18)$$

Surface pressure, π, versus log C_{Saq} plots were used to determine A_D from the quasi linear slope of the Gibbs adsorption isotherm (eq. 15). K_{aw} was then determined by means of the Szyszkowski equation (eq.18) using the cross-sectional area of the molecule, A_D, determined by means of the Gibbs equation. Measurements of the surface pressure, π, as a function of the drug concentration, C_{Saq} moreover yield the critical micelle concentration, CMC and the solubility limit of the compound [62, 74].

Partition Coefficients. The drug concentration in the lipid bilayer or the membrane mimicking system, C_{Sm}, is given by

$$C_{Sm} = C_{Saq} \cdot K_{lw}, \quad (19)$$

where C_{Saq} is the drug concentration in aqueous solution and the partition coefficient K_{lw} quantifies drug partitioning between the aqueous phase and the lipid bilayer (or the lipid mimicking phase). Depending on the purpose of the measurement the drug concentration in the membrane, C_{Sm}, is given in mole fraction units [moles/mole lipid] or in [moles/liter lipid]. The drug concentration in the aqueous phase, C_{Saq}, is generally given in [moles/liter] but mole fraction units are also used. The resulting partition coefficients are summarized in Table **1**.

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