Pumping of drugs by P-glycoprotein: a two-step process?

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ABSTRACT

The apparent inhibition constant, $K_{app}$, for the blockade of P-glycoprotein (P-gp) by four drugs, verapamil, cyclosporin A, XR9576, and vinblastine, was measured by studying their ability to inhibit daunorubicin and calcein-AM efflux from four strains of Ehrlich cells with different levels of drug resistance and P-gp content. For daunorubicin as a transport substrate, $K_{app}$ was independent of [P-gp] for verapamil but increased strictly linearly with [P-gp] for vinblastine, cyclosporin A and XR9576. A theoretical analysis of the kinetics of drug pumping and its reversal shows that $K_{app}$ for inhibition should increase linearly with the amount of pumps present in the membrane for a reverser that inhibits pumping from the cytoplasmic face. In contrast, if the reverser acts by blocking transport from the outer face, i.e. pre-emptively, $K_{app}$ should be independent of the number of pumps present. The experimental data suggest that verapamil blocks pumping at the extracellular face of the membrane whereas the other three blockers act on pumping from the cytoplasmic phase. The maximum degree of inhibition was the same for all four blockers, thus they do not act in parallel but, rather, in serial fashion, i.e. a drug that is pumped from the cytoplasmic phase has to pass the pre-emptive route upon leaving the cell. Our results are consistent with the Sauna-Ambudkar two-step model for pumping by P-gp. We suggest that the vinblastine/cyclosporin A/XR9576-binding site accepts daunorubicin at the cytoplasmic face and transfers it to the verapamil-binding site, from where daunorubicin is emptied at the extracellular surface.
Multidrug resistance (MDR) constitutes a major barrier to effective chemotherapy of cancer (Bates et al., 1996). Although alternate resistance mechanisms exist (Litman et al., 2001) (Sparreboom et al., 2003; Borst et al., 2000), the MDR phenotype is most often characterized by a reduced intracellular drug level and overexpression of the ABC transporter P-glycoprotein (P-gp) that is capable of transporting a wide range of xenobiotic compounds out of tumor cells (Chabner and Fojo, 1998; Seelig and Landwojtowicz, 2000). The transport activity of P-gp can be blocked and multidrug resistance reversed by the addition of substrate analogues, or so-called chemosensitizers or modulators (Skovsgaard et al., 1984; Krishna and Mayer, 2001).

In the literature, variable values for IC$_{50}$ (the drug concentration that gives half-maximal inhibition) for P-glycoprotein reversal are reported for the same drug in different models. For example, for the blocker Cyclosporin A, the IC$_{50}$ for inhibition of daunorubicin pumping by P-gp ranges from 0.04 µM (P388/ADR cells) (Ayesh et al., 1996) to ca. 4 µM (CEM/vinblastine1000 cells) (Hu et al., 1990), with a range of intermediate values (Litman et al., 1997; Lan et al., 1996; Cardarelli et al., 1995). For verapamil, the IC$_{50}$ for reversal of daunorubicin extrusion by P-gp ranges from 0.4 µM (SW2780AD cells) (Javaheri et al., 1983) to 10 µM (CEM/vinblastine1000 cells) (Hu, de Luise, Martin, and Zalcberg, 1990), and the in vivo situation is further complicated as oxidative metabolites may act as substrates and inhibitors of P-gp (Pauli-Magnus et al., 2000).

Part of the variability can be ascribed to different measures of the reverser’s effect on drug pumping, either by assessing the effect of the blocker on reversing resistance to cytotoxicity (Lee et al., 1994), or on reversing the drug accumulation deficit due to P-gp (Lan, Ayesh, Lyubimov, Pashinsky, and Stein, 1996). The discrepancy remains, however, even when measurements are made using the same procedure. Thus, Hu et al (Hu, de Luise, Martin, and Zalcberg, 1990) measured the effect of Cyclosporin A and verapamil on daunorubicin accumulation in two resistant variants of CEM/CCRF cells and found, that for Cyclosporin A, IC$_{50}$ increased from 1.7 µM in the moderately resistant subline CEM/vinblastine100 to 4 µM for the highly resistant CEM/vinblastine1000 line. For another P-gp blocker, GF120918, Chen et al (Chen et al., 2000) found that its IC$_{50}$ for reversal of Tc-
Tetrofosmin transport increased from 40 nM to 385 nM for a low P-gp expressing and a high P-gp expressing drug resistant carcinoma cell line, respectively. In both these studies the higher IC₅₀ was found for the more resistant cell line. We wondered if there was some casual relationship between the rise in IC₅₀ and the increasing number of pump molecules. Therefore, we asked the question: Does IC₅₀ for a reverser vary with the pump/leak ratio, and what might be the basis for this?

In our previous studies, we measured drug accumulation and P-gp content in four Ehrlich ascites tumor cell (EATC) lines selected for increasing drug resistance (Litman, Nielsen, Skovsgaard, Zeuthen, and Stein, 1997). We found that daunorubicin accumulation varied inversely with the P-gp content of the cell. Thus, these four EATC lines (termed P6, P12, P36, and P72, possessing increasing levels of P-gp) provide a unique opportunity for testing whether IC₅₀ values for blockers do in fact vary with the pump/leak ratio and, if they do so, is this in all circumstances, or only with certain substrates? We studied this experimentally by measuring the blockade of P-gp using two substrates and a number of reversers.

We first present the theory for the relation between IC₅₀ and the pump-leak ratio in different pumping models, and then follow our experimental studies, designed around this theory.
Methods

Tumor cells

Five different Ehrlich cell lines were used for the drug accumulation experiments: The parental, drug sensitive tumor, EHR2, and cells from the daunorubicin resistant subline EHR2/0.8, maintained with daunorubicin 0.8 mg/kg × 4 weekly (50% of the LD_{10} dose). Cells from passages 6, 12, 36 and 72, in the following termed P6, P12, P36 and P72 were used. All cells were maintained in vivo in NMRI/DBA mice after intraperitoneal inoculation. No drug was administered in the last passage before the experiments. These cell lines have previously been characterized in our laboratories with respect to drug resistance (Nielsen et al., 1994), kinetics of daunorubicin transport (Nielsen et al., 1995), lipid composition (Litman et al., 1995), ATPase activity (Litman, Nielsen, Skovsgaard, Zeuthen, and Stein, 1997), pH regulation (Litman et al., 1998), and taurin uptake (Poulsen et al., 2002).

P-gp expression

The P-gp content was quantified by Western blot analysis as described in detail in (Nielsen, Maare, and Skovsgaard, 1994). In essence, membrane-enriched pellets were separated by SDS-PAGE, and the protein was transferred to nitrocellulose membranes, which were incubated overnight with C219 anti-P-gp antibody (Signet, MA). The P-gp specific bands were scanned on a densitometer (Shimadzu, Kyoto, Japan), and the relative concentration of P-gp was calculated using a standard curve composed of membrane preparations with defined amounts of P-gp.

Daunorubicin accumulation

The uptake of 5 μM daunorubicin was followed with time (5, 15, 29, 45, 60 min) at 37 °C, essentially as described by Skovsgaard (Skovsgaard, 1978), and the effect of drugs on the steady-
state accumulation of daunorubicin (at time 60 min) was investigated. To the incubation medium
(NaCl, 57 mM; KCl, 5 mM; MgSO₄, 1.3 mM; NaH₂PO₄, 9 mM; Na₂HPO₄, 51 mM; fetal calf serum, 5% (v/v); pH 7.45) was added glucose to 10 mM and the test drug: either verapamil, cyclosporin A, vinblastine, or XR9576, at various concentrations. Energy deprivation experiments without glucose but including 10 mM sodium azide were performed to obtain an estimate of the maximum accumulation level of daunorubicin. The uptake of daunorubicin was quantitated fluorometrically in a Hitachi F3010 spectrofluorometer (Tokyo, Japan) with excitation wavelength 470 nm, and emission wavelength set to 585 nm. The kinetic parameter for half-maximal inhibition of pumping by P-gp, K_{app}, was obtained by curve-fitting using the Marquardt-Levenberg algorithm (SigmaPlot, SPSS Science, Chicago, IL).

**Calcein accumulation**

Calcein accumulation was measured in a 96-well plate assay modified after Eneroth et al. (Eneroth et al., 2001). Ehrlich cells were harvested, washed and resuspended in 100 µl transport medium in 96-well microtiter plates. Next, the test drug (cyclosporin A, verapamil, vinblastine, or XR9576) at various concentrations was added to each well. After a 15 min preincubation, calcein-AM was added to 250 nM, and the plate was incubated at 37 °C for 60 min. Calcein-specific fluorescence was measured in a Bio-Tek FL600 (Boule Nordic, Denmark) microplate fluorescence reader with 485/20 nm excitation and 530/25 nm emission filters. For each test drug, the calcein accumulation (in arbitrary fluorescence units, AFU) was plotted versus drug concentration. The kinetic parameter for half-maximal inhibition of pumping by P-gp, K_{app}, was obtained by curve-fitting using the Marquardt-Levenberg algorithm (SigmaPlot, SPSS Science, Chicago, IL).
Chemicals

XR9576 was kindly provided by Dr. Susan Bates (National Cancer Institute, NIH, Bethesda, MD). All other chemicals were of analytical grade, purchased either from Merck (Darmstadt, Germany) or from Sigma (St. Louis, USA).
Theory

Figure 1A is a cartoon depicting the steady-state accumulation of a P-gp substrate which is pumped out from the cytoplasmic phase (where it is at concentration $S_i$) by a pump, which is operating at a velocity $k_i$, against a bidirectional leak with rate $p$. [In the following treatment, the subscript $i$ will be used to denote processes occurring at the \textit{inner} phase of the membrane, while subscript $o$ denotes processes at the \textit{outer} surface]. The pump is inhibited by a blocker having the intrinsic affinity $K_i$ for the pump and being at concentration $I$. The following analyses are in terms of the concentration of free substrate. If a substrate is bound to some intracellular component (e.g. DNA, lipids, or trapped inside lysosomes) the concentration measured is the total concentration, which will be proportional to the free concentration where this is below saturation of the binding component.

The ratio of free substrate concentrations (inside to outside) at steady-state is given by:

$$\frac{S_i}{S_o} = \frac{p}{p + k_i}$$

as elaborated on by Stein (Stein, 1997). When substrate permeability is very high in comparison with the pump rate, i.e. $p >> k_i$, the ratio of $S_i/S_o$ approaches unity. Thus, it appears that even if drug is rapidly pumped out of the cell, an even faster back-diffusion will overwhelm the pump. The concept of ‘fast’ versus ‘slow’ diffusing agents has been comprehensively reviewed by Gera Eytan and coworkers (Eytan et al., 1996) (Eytan and Kuchel, 1999), and the influence of passive permeability on the kinetics of P-gp has subsequently been demonstrated experimentally (Lentz et al., 2000; Westerhoff et al., 2000; Wielinga et al., 2000).

The fraction of pumping activity in the presence of $I$ is:

$$\frac{K_i}{K_i + I} \quad \text{...eq. 1}$$
It simple to show that at the steady-state:

\[
\frac{S_i}{S_o} = \frac{p}{p + k_i} = \frac{p}{p + k_{i,0} \frac{K_i}{K_i + I}}
\]

…eq. 2

where \(k_{i,0}\) is the uninhibited pump rate.

Figure 1B is computed from equation 2 where \(S_i/S_o\) is plotted at different inhibitor concentrations \(I\) for 5 different ratios of the pumping rate constant \(k_{i,0}\) to the leak rate \(p\).

Figure 1C shows the steady-state level of a P-gp substrate that is pumped out pre-emptively, that is, en route across the cell membrane, as the compound enters the extracellular leaflet of the membrane bilayer. Here, the ratio of free substrate concentrations (inside to outside) at steady-state is given by:

\[
\frac{S_i}{S_o} = \frac{p - k_o}{p}
\]

Thus, if substrate permeability is very high in comparison with the pump rate, i.e. \(p >> k_o\), the ratio of \(S_i/S_o\) approaches unity. Thus, in this case too, even if drug is rapidly pumped out of the cell, back-diffusion will dominate and the effect of pumping will not be seen.

The pump is again inhibited by a blocker having intrinsic affinity \(K_o\) for the pump and being at concentration \(I\) (equation 1 still applies).

For this case, at the steady-state.

\[
\frac{S_i}{S_o} = \frac{p - k_o}{p} = \frac{p - k_{i,0} \frac{K_o}{K_o + I}}{p}
\]

…eq.3

Figure 1D is now computed from equation 3 where \(S_i/S_o\) is plotted as a function of the inhibitor concentration \(I\) for 4 different ratios of the pumping rate constant \(k_{i,0}\) to the leak rate \(p\). For this case a constraint exists: \(k_o \leq p\), i.e. the pump cannot extract from the membrane more than enters it.
Visual inspection of figures 1B and 2B suggests that in the former case, the apparent half-saturation concentration \( K_{\text{app}} \) (i.e. the observed IC\(_{50}\)) increases with the rate of pumping, \( k_{i,0} \), whereas in the latter case the \( K_{\text{app}} \) does not depend on the pumping rate \( k_o \).

Mathematically, (see the Appendix) equation 2 can be solved to yield:

\[
K_{\text{app}} = K_i \frac{p + k_{i,0}}{p} \quad \text{...eq.4}
\]

Thus, in the case of a pump acting from the cytoplasmic phase, the half-saturation concentration for the effect of a blocker on the steady-state accumulation level (\( K_{\text{app}} \)) depends on the rate of pumping, that is on the number of pumps present in the cell membrane.

If equation 3 is similarly treated, (see the Appendix) one obtains:

\[
K_{\text{app}} = K_o. \quad \text{...eq.5}
\]

That is, for pre-emptive pumping, the apparent coefficient for inhibition by a blocker equals the intrinsic \( K_o \).

Finally, one can analyze the situation where the pump acts both on substrate present in the inner layer and outer layer of the membrane.

For this case, at the steady-state.

\[
\frac{S_i}{S_o} = \frac{p - k_o}{p + k_i} = \frac{p - k_{i,0} \frac{K_o}{K + I}}{p + k_{i,0} \frac{K}{K + I}} = \frac{p - k_{i,0}}{p + k_{i,0}} \frac{K_o}{K + I} \quad \text{...eq.6}
\]

Again, the constraint \( k_o \leq p \) applies.

Figure 1E shows how \( S_i/S_o \) varies with the inhibitor concentration \( I \) at 4 different ratios of the pumping rate constant \( k \) to the leak rate \( p \). Here, for simplicity, we have assumed that the properties of the pumping system are the same at both faces of the membrane; that is, that the affinity and the rate of pumping do not differ at the two faces.
Visual inspection and computer fitting of these predicted curves to a Michaelis-Menten kinetics model, shows that in this case, the apparent $K_{app}$ increases with the pump rate, but to a maximum which is two-fold the value at limiting low concentrations of the inhibitor.
Results

We have attempted to apply the theoretical analysis derived above, to an experimental system, where we have a series of cell lines with different abilities to accumulate drugs and with measured (and differing) amounts of P-gp. This is equivalent to varying the pump rate $k_{e,o}$ in the different cells, assuming that the pump rate is proportional to the amount of P-gp.

Figure 2A depicts the steady-accumulation of daunorubicin as a function of verapamil concentration for the four different EAT cell lines. The curves were fitted to the Michaelis-Menten equation

$$V = V_{max} \frac{I}{I + K_{app}} + V_0$$

...eq. 7

where $V$ is the amount of daunorubicin accumulated at a concentration $I$ of blocker, $V_0$ is the intracellular daunorubicin level in the absence of blocker, $V_{max}$ is the daunorubicin accumulation at maximum concentration of blocker, and $K_{app}$ is the blocker concentration which brings the daunorubicin accumulation to half-way between the value at $I=0$, and that at $I=\infty$.

Figure 2B is a similar plot showing the effect of vinblastine used as a blocker of daunorubicin transport, and Figure 2C shows a parallel experiment with the high-affinity blocker XR9576. Table 1 records the values of $K_{app}$ obtained by curve-fitting for these three blockers as well as for cyclosporin A where similar data were obtained (not shown).

It would appear that for the blockers, XR9576, cyclosporin A, and vinblastine the $K_{app}$ increases with the amount of P-gp.

Figure 3 is a plot of $K_{app}$ vs the P-gp level for all these blockers, where the $K_{app}$ values have been normalized to the value found at the lowest P–gp concentration. The regression line through the data has $r^2 = 0.90$. The values of $K_{app}$ at the highest values of P-gp content were close to 4 times the value.
at the lowest resistance level. In contrast, (see Table 1) the $K_{\text{app}}$ for verapamil as a blocker does not appear to increase with the P-gp level.

By transforming eq.4 one can obtain the result that

$$K_i = K_{\text{app}} \frac{p}{p + k_{i,0}}$$

Now, $p / (p+k_{i,0})$ is simply the ratio of unblocked to blocked accumulation of daunorubicin, which can be found from the extrapolates to zero and infinite blocker of the curves of figure 2 and the similar data for cyclosporin A.

Table 1 records also these estimates of $p/(p+k_{i,0})$ for each blocker, and the corresponding values of $K_i$, calculated from $K_{\text{app}}$ and the corresponding value of $p/(p+k_{i,0})$. It appears that the derived values of $K_i$ do not vary with increasing amounts of P-gp. The mean of the recorded values of $K_i$ for each blocker can therefore be taken as an estimate of the $K_i$ for that blocker in its action of inhibiting P-gps function from the cytoplasmic surface.

Similar sets of data were obtained for calcein accumulation in the presence of these four blockers. Figure 4 depicts the data gathered for the blockers verapamil, vinblastine and cyclosporin A. The values for $K_{\text{app}}$ obtained by curve-fitting to eq.7 are collected in Table 1 together with the parameters found for XR9576. For none of these blockers does $K_{\text{app}}$ increase significantly with the amount of P-gp, in contrast to the situation found for daunorubicin with the blockers cyclosporin A, XR9576, and vinblastine.
Discussion

In the theory section it was shown that $K_{app}$, the half-inhibition constant for the action of a blocker on a drug transport pump, increases with the amount of pumps present if pumping is occurring from the cytoplasmic phase. In contrast, if pumping occurs pre-emptively, $K_{app}$ is independent of the number of transporters. Chen et al (Chen, Luker, Dahlheimer, Pica, Luker, and Piwnica-Worms, 2000) studied the action of the blocker GF120918 on the accumulation of the P-gp substrate Tc-Tetrofosmin in drug resistant epidermoid carcinoma cell lines. It was found that the $K_{app}$ values for GF120918 were 40 nM and 385 nM for a moderately (low P-gp expression) and a highly (high P-gp expression), resistant cell line, respectively. This would be understandable if the effect of GF120918 was to block the pump at the cytoplasmic surface. Our data (Fig. 2 and Table 1) show that for the accumulation of the P-gp substrate daunorubicin, the $K_{app}$ for vinblastine, cyclosporin A, and XR9576, increase with the amount of P-gp expression (Fig. 3). This suggests that the action of these blockers could also be on the cytoplasmatic side of the cell membrane (Fig. 1A). If this interpretation is correct, one would be entitled to compute the intrinsic affinity of blocker for the pump, $K_i$, from the several values of Kapp and the corresponding value for the steady-state accumulation ratio for each cell line. The estimates of $K_i$ are collected in Table 1 for cyclosporin A, XR9576, and vinblastine.

In contrast, for verapamil $K_{app}$ does not appear to increase with P-gp expression, suggesting that verapamil interferes with pre-emptive pumping (Fig. 1C). Thus, the $K_i$ for verapamil is given directly by the measured value of $K_{app}$ (Table 1).

The situation with calcein-AM appears more complex. Here (Fig.4 and Table 1), for all the blockers $K_{app}$ does not seem to increase with P-gp expression. It could be that calcein-AM itself is pre-emptively pumped out of the membrane, differing in this way from daunorubicin. This would be in accordance with the suggestion of Homolya et al (Homolya et al., 1994), the pioneers in the use of
calcein-AM as a P-gp substrate: namely, that calcein-AM and other fluorescent methyl esters are extruded directly from the cell membrane, before reaching the cytoplasmic phase. Pre-emptive pumping of calcein-AM is also in accordance with the theoretical analysis of Stein (Stein, 1997), who showed that the initial rate of substrate accumulation is reduced by pumping only if such pumping is pre-emptive. Calcein-AM uptake is always measured as an initial rate, since free calcein-AM does not accumulate within the cell, calcein-AM being cleaved by intracellular esterases to free calcein as soon as it enters the cell (Holló et al., 1994). Since the blockers do affect the initial rate of calcein accumulation and, hence, the rate of calcein-AM extrusion, they must be affecting the initial rate of the process.

If this interpretation is correct, that the effect of blockers on daunorubicin accumulation can occur at the cytoplasmic surface, whereas the effect on calcein accumulation is on pre-emptive pumping, then one might be able to understand why the intrinsic $K_i$ values for cyclosporin A, vinblastine, and XR9576 found for daunorubicin accumulation differ markedly from the values found for calcein accumulation (Table 1). This finding would imply that the intrinsic affinity of the blockers at the cytoplasmic surface is some 4 times higher than the affinity at the outer half of the bilayer. For verapamil as a blocker, on the other hand, the $K_{app}$ value for its action on daunorubicin accumulation is not different from its value for blocking calcein-AM extrusion. This would be expected if both measures were of the same process, namely pre-emptive pumping of substrate.

Sauna and Ambudkar have proposed a two-step model for the catalytic cycle of P-gp (Sauna and Ambudkar, 2001). Here, drug is bound at a high-affinity site at the cytoplasmic surface, and then moves to a low-affinity site, from which it is extruded to the extracellular medium. It is tempting to identify the high-affinity blocker site that our analysis suggests at the cytoplasmic surface with the high-affinity site for drugs postulated in the Sauna & Ambudkar model. The low-affinity site for blockers, which our analysis suggests is concerned in pre-emptive pumping, might correspond to the
low-affinity drug-binding site in the above model. This interpretation is also supported by transport data obtained with a series of human lymphoblastoid cell lines (CEM wt, CEM ADR5000, and CEM Col1000); here, the affinity of verapamil to P-gp differed at the two sides of the plasma-membrane (Köhler and Stein, 2003).

What still has to be accounted for is that verapamil, which we postulate is blocking at the pre-emptive site, can block all of the effect of the pump on daunorubicin accumulation, that is, both from the cytoplasmic surface and the pre-emptive pumping. This would be expected if the pathway for drug extrusion occurs by a serial process, i.e., drug which is being pumped from the cytoplasmic phase has to go through the pre-emptive passage on its way out of the cell (Figure 5). However, an alternative explanation of the experimental results could be, that verapamil, being a fast diffusing drug, is likely to quickly partition across the membrane, and accumulate also in the cytosolic leaflet of the membrane (which is rich in negatively charged lipids that would tend to interact electrostatically with the cationic compound), thus, exerting its inhibitory effect at both sites of the membrane. That the biophysical properties of membrane lipids are affected by chemosensitizers such as verapamil is suggested in a recent review by Hendrich and Michalak (Hendrich and Michalak, 2003).
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Appendix

Derivation of

\[ K_{\text{app}} = \frac{p + k_{i,0}}{p} \]

For pumping from the cytoplasmic face \( S_i/S_o = \frac{p}{p + k_i} \).

At zero concentration of blocker (I=0) this becomes \( S_i/S_o = \frac{p}{p + k_{i,0}} \).

At infinitely high concentration of I, \( S_i/S_o = 1 \), which equals \( (p + k_{i,0}) / (p + k_{i,0}) \).

Define \( \{S_i/S_o\}_{1/2} \) as the value of \( S_i/S_o \) halfway between none and complete inhibition, and \( K_{\text{app}} \) as the value of I at this \( S_i/S_o \).

Then, at \( I = K_{\text{app}} \):

\[ \{S_i/S_o\}_{1/2} = \frac{(p + k_{i,0}/2)}{(p + k_{i,0})}, \quad \ldots \text{eq A1.} \]

which is half-way between \( S_i/S_o \) at I=0, and \( S_i/S_o \) at I=\( \infty \).

Substituting \( K_{\text{app}} \) for I in eq.2,

\[ \frac{\{S_i/S_o\}_{1/2}}{p} = \frac{p}{p + k_{i,0}} \frac{K_i}{K_i + K_{\text{app}}} \quad \ldots \text{eq.A2} \]

Equating the right-hand sides of equations A1 and A2, and solving for \( K_{\text{app}} \) in terms of \( p \) and \( k_{i,0} \), we obtain:

\[ K_{\text{app}} = K_i \frac{p + k_{i,0}}{p} \]
Derivation of:  

\[ K_{\text{app}} = K_o \quad \text{...eq.5} \]

For pre-emptive pumping \( S_i/S_o = (p-k_o) / p \).

At zero concentration of blocker (I=0) this becomes \( S_i/S_o = (p - k_{o,0}) / p \)

At infinitely high concentration of I, \( S_i/S_o = 1 \), which equals \( p / p \).

Again, define \( \{S_i/S_o\}_\text{½} \) as the value of \( S_i/S_o \) halfway between none and complete inhibition, and \( K_{\text{app}} \) as the value of I this \( S_i/S_o \).

Then, at \( I = K_{\text{app}} \):

\[ \{S_i/S_o\}_\text{½} = (p - k_{o,0}/2) / p \quad \text{...eq A3.} \]

which is half-way between \( S_i/S_o \) at I=0, and \( S_i/S_o \) at I=\( \infty \).

Substituting \( K_{\text{app}} \) for I in eq.3,

\[ \frac{S_i}{S_o} = \frac{p - k_{o,0}}{K_o + K_{\text{app}}} \quad \text{...eq.A4} \]

Again, equating the right-hand sides of equations A3 and A4, and solving for \( K_{\text{app}} \), we obtain:

\[ K_{\text{app}} = K_o \]
References


FOOTNOTES

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List of abbreviations:

I, inhibitor concentration

IC$_{50}$, the drug concentration that gives half-maximal inhibition (e.g. of P-glycoprotein)

$K_{app}$, the apparent half-saturation concentration (e.g. IC$_{50}$)

$k_i$, the pump rate for pumping from the inside

$k_{i,0}$, the uninhibited pump rate for pumping from the inside

$k_o$, the pump rate for (pre-emptive) pumping from the outer face of the membrane

$k_{o,0}$, the uninhibited pump rate for pumping from the outer face of the membrane

$K_i$, intrinsic affinity for P-gp at the inner leaflet of the cell membrane

$K_o$, intrinsic affinity for P-gp at the outer face of the membrane (pre-emptive pumping)

$p$, the leak rate of a compound that crosses the membrane by passive diffusion

$[P\text{-gp}]$, concentration of P-glycoprotein

$S_i$, intracellular substrate concentration

$S_o$, extracellular substrate concentration

MDR, Multidrug resistance
Legends for Figures

**Figure 1.** Three models for drug pumping by P-glycoprotein and their theoretical predictions:

(A) and (B): Pumping is from the cytoplasmic face of the membrane. (A), cartoon. The bidirectional leak rate constant is p, and the pump rate k_i. I is the blocker. The curves in (B) give the predictions of this model (see text, eqtn 2) with pump/leak ratios set at 0.1, 1, 5, 10, and 100. The y-axis is the drug distribution ratio, the x-axis is the concentration of blocker where the value of K_i (see text) is set at 10.

(C) and (D): Pumping is pre-emptive, i.e., drug is pumped out on its way into the membrane. (C), cartoon. Symbols as in A. The curves in (D) give the predictions of this model (see text, eqtn 3) with pump/leak ratios set at 0.1, 1, 5, and 10, and K_o set at 10.

(E) Pumping is both pre-emptively and from the cytoplasmic face of the membrane. The curves give the predictions of the membrane-symmetric case of this model (see text, eqtn 6) with pump/leak ratios set at 0.1, 1, 5, and 10, and K_i = K_o set at 10.

**Figure 2.** Experimental tests of the theoretical models of Fig 1, using daunorubicin (DNR) as substrate. Accumulation of daunorubicin was measured during 60 min as described in Materials and Methods. Increasing levels of three blockers were used on four EATC lines, as indicated. In (A), the blocker was verapamil, in (B) vinblastine and in (C) XR9576. The lines are the best-fits to the modified Michaelis-Menten equation as given in the text, eqtn 7.
Figure 3. $K_{\text{app}}$ values derived from the curve-fits of Fig 2 for the blockers cyclosporin A, XR9576 and vinblastine plotted against the P-gp level of the four cell lines studied.

Symbols are: ○ Cyclosporin A; ▲ Vinblastine; ● XR9576.

In each case, the $K_{\text{app}}$ is normalized to the value found for the P6 line set at 1.

Figure 4. Experimental tests of the theoretical models of Fig 1, using calcein-AM as substrate.

Accumulation of calcein was measured during 60 min as described in Materials and Methods.

Increasing levels of three blockers were used on four EATC lines, as indicated. In (A), the blocker was verapamil, in (B) vinblastine and in (C) cyclosporin A. The lines are the best-fits to the modified Michaelis-Menten equation as given in the text, eqtn 7.

Figure 5. Drug pumping as a serial process. Cartoon depicting daunorubicin being pumped across the membrane by P-gp in two-site fashion. The blockers cyclosporin A (CsA), XR9576 (XR) and vinblastine (VL) block at the cytoplasmic face, verapamil (VER) at the extracellular face (pre-emptively) but daunorubicin (DNR) must egress through the extracellular site if it is to leave the membrane.
Fig. 1.
Fig. 1.
Fig. 1.
Fig. 2.
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 4.
TABLE 1

Kinetic parameters for inhibition of daunorubicin (DNR) and calcein-AM (C-AM) efflux from four progressively resistant (P6, P12, P36, P72) Ehrlich ascites tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Drug</th>
<th>( P6 ) S/( S_0 = 0.88 )</th>
<th>( P12 ) S/( S_0 = 0.66 )</th>
<th>( P36 ) S/( S_0 = 0.46 )</th>
<th>( P72 ) S/( S_0 = 0.34 )</th>
<th>Mean ( K_i )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_{app} )</td>
<td>( K_i )</td>
<td>( K_{app} )</td>
<td>( K_i )</td>
<td>( K_{app} )</td>
<td>( K_i )</td>
</tr>
<tr>
<td>DNR</td>
<td>Verapamil</td>
<td>9.4</td>
<td>5.3 (5.3)</td>
<td>6.4 (6.4)</td>
<td>6.4 (6.4)</td>
<td>6.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Cyclosporin A</td>
<td>0.36</td>
<td>0.811</td>
<td>0.54</td>
<td>1.30</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>Vinblastine</td>
<td>1.1</td>
<td>3.6</td>
<td>2.38</td>
<td>4.7</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>XR9576</td>
<td>0.03</td>
<td>0.06</td>
<td>0.04</td>
<td>0.113</td>
<td>0.05</td>
</tr>
<tr>
<td>C-AM</td>
<td>Verapamil</td>
<td>8.0</td>
<td>6.4</td>
<td>4.4</td>
<td>4.2</td>
<td>5.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Cyclosporin A</td>
<td>1.7</td>
<td>1.6</td>
<td>1.6</td>
<td>1.8</td>
<td>1.68 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Vinblastine</td>
<td>6.5</td>
<td>5.7</td>
<td>4.7</td>
<td>5.4</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>XR9576</td>
<td>0.34</td>
<td>0.19</td>
<td>0.19</td>
<td>0.14</td>
<td>0.22 ± 0.04</td>
</tr>
</tbody>
</table>

The parameters shown are:

- \( K_{app} \) (in \( \mu \text{M} \)), the apparent inhibition constant, i.e. the concentration of blocker that gives half-maximal inhibition of DNR or C-AM efflux;
- \( K_i \) (in \( \mu \text{M} \)), the intrinsic affinity of the blocker for the pump; \( S/\( S_0 \) \), the ratio of intra- to extracellular drug concentration at steady-state.

\( ^a \) The apparent and intrinsic constants for inhibition of DNR efflux by verapamil are identical.

\( ^b \) For C-AM, the ratio \( S/\( S_0 \) \) is zero, because any C-AM that enters the cellular compartment is immediately hydrolyzed by intracellular esterases to free calcein. Therefore \( S_i \) is kept at zero concentration, while \( S_o \) remains constant.