BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Structure-activity relationships of P-glycoprotein interacting drugs: kinetic characterization of their effects on ATPase activity

Thomas Litman [a,b,*], Thomas Zeuthen [b], Torben Skovsgaard [a], Wilfred D. Stein [c]

[a] *Department of Oncology, Herlev Hospital, University of Copenhagen, Copenhagen, Denmark*
[b] *Department of Medical Physiology, The Panum Institute, University of Copenhagen, Copenhagen, Denmark*
[c] *Department of Biochemistry, Silberman Institute of Life Sciences, Hebrew University, Jerusalem, 91904, Israel*

Received 3 January 1997; revised 19 March 1997; accepted 20 March 1997

Abstract

We have determined the kinetic parameters for stimulation and inhibition by 34 drugs of the P-glycoprotein ATPase in membranes derived from CR1R12 Chinese hamster ovary cells. The drugs chosen were sets of calmodulin antagonists, steroids, hydrophobic cations, hydrophobic peptides, chemotherapeutic substrates of P-glycoprotein, and some other drugs with lower affinity for P-glycoprotein. We studied how these kinetic parameters correlated with the partition coefficient and the Van der Waals surface area of the drugs. The maximum velocity of ATPase stimulation decreased with surface area and showed a suggestion of a maximum with increasing partition coefficient. The affinity of these drugs for P-glycoprotein showed no significant correlation with partition coefficient but was highly correlated with the surface area suggesting that binding between modulators and P-glycoprotein takes place across a wide interaction surface on the protein.

Keywords: Multidrug resistance; P-glycoprotein; ATPase; Structure-activity relationship; Kinetic parameter

1. Introduction

In the preceding paper of this series (Litman et al. ATPase activity of P-glycoprotein related to emergence of drug resistance in Ehrlich ascites tumor cell lines, BBA, this issue), we characterized the modulation of the ATPase activity of P-glycoprotein derived from multidrug resistant Ehrlich ascites tumor cells for nine different drugs. In order to provide insight into the mechanism whereby P-glycoprotein interacts with its modulators we have extended this study to a total of 34 different drugs and performed a QSAR (quantitative structure-activity relationships) analysis using the molecular mechanics programs ChemPlus™ and HyperChem®. The QSAR parameters obtained are based on calculations of minimum energy conformations of the drugs, and have been compared to parameters which describe the affinity for and stimulation of the P-glycoprotein ATPase. It has been suggested that effective MDR modulators share physical properties such as octanol/water partitioning [1] and molecular size [2] and shape [3,4]. Partitioning is of key importance in vivo behavior of xenobiotics [5], and is most often described by log P (reviewed by Leo et al. [6]), but also other parameters reflecting

* Corresponding author. Department of Medical Physiology, The Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark. Fax: (45)-3532 7420. Email: T.Litman@mfi.ku.dk

PII S0925-4439(97)00026-4

bulk, such as molecular weight, volume, refractivity, and Van der Waals surface area [7] have proven to be important variables in QSAR analysis. All these parameters have been calculated for the test panel of drugs.

In the present study, the source of P-glycoprotein was the microsomal membrane fraction prepared from a highly drug resistant, P-glycoprotein-overproducing CHO cell line, CR1R12. This cell line constitutively overexpresses P-glycoprotein, which amounts to a maximum of 32% (w/w) of the total plasma membrane protein [8]. Thus, the CR1R12 cell line provides an excellent source of membranes enriched in P-glycoprotein, and enables a characterization of the P-glycoprotein associated ATPase activity.

For all ATPase assays, the *microsomal* membrane fraction of CR1R12 cells was used with no further purification or reconstitution steps. This approach was chosen for five reasons. First, the signal-to-noise ratio was sufficiently high in the microsomal fraction to obtain an adequate drug-modulatable ATPase activity pattern. Second, in the crude membrane fraction, the activity of P-glycoprotein is assayed in its genuine, 'natural' microenvironment, with minimal risk of perturbation of the system. Third, it is now known that some of the common protease inhibitors employed in reconstitution, such as leupeptin and pepstatin A, themselves are substrates of P-glycoprotein [9] and thus would tend to pre-activate the P-glycoprotein ATPase. Fourth, the major problem in any reconstitution procedure: the use of detergents (e.g. deoxycholate, digitonin, SDS, Triton X-100, CHAPS) which have been shown to inactivate P-glycoprotein, even at very low concentrations [10,11], is avoided. Fifth, the introduction of an artificial lipid environment, the composition of which can greatly affect the characteristics of reconstituted P-glycoprotein [11–14], is evaded by using the P-glycoprotein enriched, microsomal membrane fraction.

We have characterized the kinetic parameters describing the activation and inhibition of the P-glycoprotein ATPase for all 34 drugs and have analyzed how the parameters for activation depend on the partition coefficient and Van der Waals surface area of the various drugs. Our results can form the basis of a strategy for improving the design of modulators of P-glycoprotein with the aim of developing more effective cancer chemotherapeutics.

2. Materials and methods

2.1. Tumor cells

The Chinese Hamster Ovary cell line, CHO CR1R12 (kindly made available for us by Dr Alan E. Senior, University of Rochester Medical Center, New York, USA), was used. The cells were maintained in α-MEM (minimal essential medium) containing 10% fetal calf serum and 5 μg/ml colchicine. In order to obtain a high yield of cells for the subsequent membrane preparation, the cells were grown in 'cell-factories' (Nunc, Roskilde, Denmark), and were harvested by a standard trypsinization procedure.

2.2. Isolation of microsomal membranes

The cells were washed with Ringer's medium (NaCl, 148.7 mM; K_2HPO_4, 2.55 mM; KH_2PO_4, 0.45 mM; $MgSO_4$, 1.2 mM; pH 7.4) before being resuspended in 10 ml lysis buffer (Hepes-Tris, 10 mM; EDTA, 5 mM; EGTA, 5 mM; dithiothreitol, 2 mM; pH 7.4) containing protease inhibitors (phenylmethylsulfonyl fluoride, 2 mM; aprotinin, 5 μg/ml; pepstatin, 10 μg/ml; leupeptin, 10 μg/ml). This and all subsequent steps were performed at 4°C. Cells were lysed by nitrogen cavitation (Parr Instrument Co., Moline, IL, USA) at 1200 psi for 20 min. Nuclei were sedimented by centrifugation at $300 \times g$ for 10 min, and subsequently mitochondria were removed by centrifugation at $4000 \times g$ for 10 min. In a third and final centrifugation ($45\,000 \times g$, 60 min) the microsomal fraction was sedimented. The pellet was resuspended in 1 ml of lysis buffer and homogenized by aspiration ten times through a 27 gauge syringe. Aliquots of 150 μl (containing 1–2 mg protein/ml) were frozen in $N_{2(l)}$ and stored at −80°C.

2.3. ATPase activity assay

The P-glycoprotein associated ATPase activity was determined according to Borgnia et al. [15], by quantitating the release of inorganic phosphate from ATP. The microsomes were thawed on ice before diluting to a protein concentration of 20 μg/ml in ice-cold ATPase assay medium (ATP, 3 mM; KCl, 50 mM; $MgSO_4$, 2.5 mM; dithiothreitol, 3 mM; Tris-HCl, 25 mM; pH 7.0) containing 0.5 mM EGTA (to inhibit

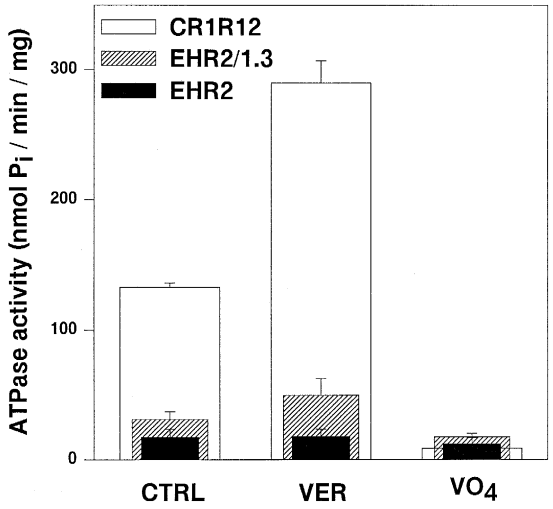

Fig. 1. ATPase activity of microsomes prepared from sensitive (EHR2) and multidrug resistant Ehrlich (EHR2/1.3) and CHO (CR1R12) cells. The bars indicate the rate of ATP hydrolysis (nmol/min/mg protein) in the absence of drug (CTRL), with 25 μM verapamil (VER), or 500 μM vanadate (VO_4) in the incubation medium (the vanadate bars are representative for experiments both with and without verapamil). Values are means ± S.E.M. ($n = 3–6$).

Ca-ATPases), 2 mM ouabain (to inhibit the Na/K-ATPase), and 3 mM azide (to inhibit mitochondrial ATPase). Each series of experiments was carried out in a 96-well microtiter plate, with reaction volumes of 50 μl/well corresponding to 1 μg protein/well. Incubation with the various drugs was started by transferring the plate from ice to 37°C for 1 h, and terminated by addition of 200 μl ice-cold stopping medium (ammonium molybdate, 0.2% (w/v); sulfuric acid, 1.3% (v/v); SDS, 0.9% (w/v); trichloroacetic acid, 2.3% (w/v); freshly prepared ascorbic acid, 1% (w/v)) to each well. After 30 min incubation at room temperature, the released phosphate was quantitated colorimetrically in a micro-plate reader (Bio-Tek Instruments, VT USA) at 620 nm. Samples incubated with 500 μM vanadate (inhibiting the P-glycoprotein ATPase) were obtained in parallel and subtracted from the measurements. Water-insoluble drugs were dissolved in ethanol or DMSO, the solvent constituted max. 1% (v/v) of the total volume.

2.4. Chemicals

All chemicals were of analytical grade, purchased either from Merck (Darmstadt, Germany) or from Sigma (St. Louis, USA). D-verapamil and ±-verapamil were kindly provided by Knoll AG (Ludwigshafen, Germany), PSC-833 was a kind gift from Sandoz (now Novartis, Basel, Switzerland). All

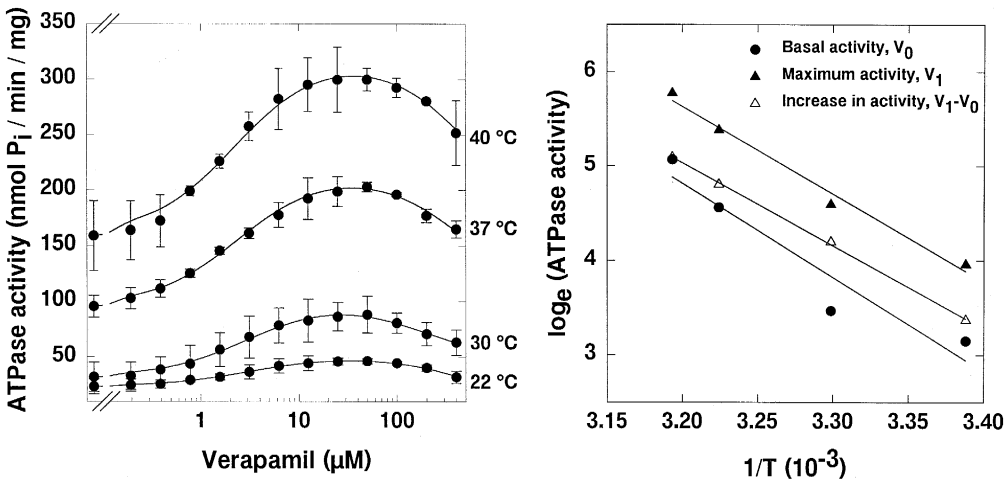

Fig. 2. P-glycoprotein ATPase activity as a function of temperature. Left panel, D-verapamil activated ATPase activity of CR1R12 microsomes at 22, 30, 37, and 40°C. The lines drawn are fitted to a modified form of the Michaelis-Menten equation taking into account both the ascent and the decline of the ATPase activity curve. Right panel, the corresponding Arrhenius plot: $\ln k = \ln A - (E^*/RT)$. $\log_e$ of the basal activity, V_0, the maximum stimulated activity, V_1, and the increase in activity, $V_1 - V_0$, as a function of the reciprocal of the absolute temperature is depicted. The lines are first order regressions to the data, the slopes of which give the activation energies: $E^*_{V1} = 77.2$ kJ/mol, and $E^*_{V1-V0} = 72.9$ kJ/mol.

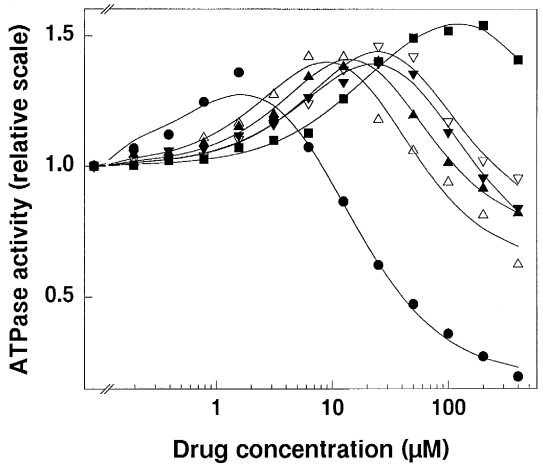

Fig. 3. Effect of calmodulin antagonists on the ATPase activity of P-glycoprotein: pimozide (●), trifluoperazine (△), fluphenazine (▲), triflupromazine (▽), chlorpromazine (▼), and promethazine (■). For clarity, the error bars are hidden.

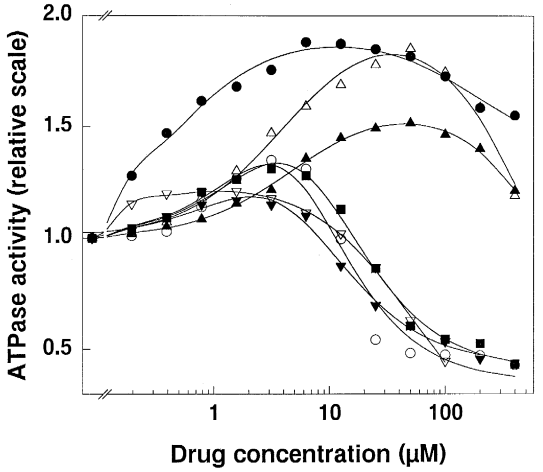

Fig. 5. Effect of hydrophobic cationic drugs on the ATPase activity of P-glycoprotein: dipyridamole (●), amiodarone (○), propafenone (△), quinidine (▲), reserpine (▽), terfenadine (▼), and mefloquine (■).

other chemicals were of analytical grade, purchased either from Merck (Darmstadt, Germany) or from Sigma (St. Louis, USA).

3. Results

3.1. Specific ATPase activity

In the preceding paper of this series (Litman et al., ATPase activity of P-glycoprotein related to emergence of drug resistance in Ehrlich ascites tumor cell lines, BBA, this issue) we studied the ATPase activity of microsomes prepared from a sensitive and five progressively resistant Ehrlich tumor cell lines. Fig. 1 shows the ATPase activity of membranes derived from two members of this series together with corresponding data from a highly P-glycoprotein expressing CHO cell line, CR1R12. The data are for membranes in the absence of any additive (CTRL), in the presence of 25 μM verapamil (VER), or 500 μM

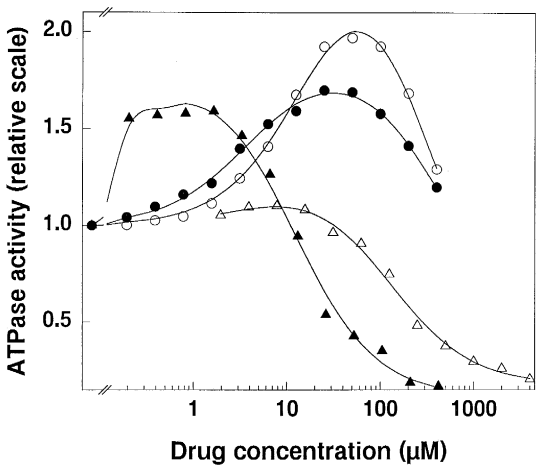

Fig. 4. Effect of steroids and a hormonal analogue on the ATPase activity of P-glycoprotein: progesterone (○), spironolactone (●), fucidin (△), and tamoxifen (▲).

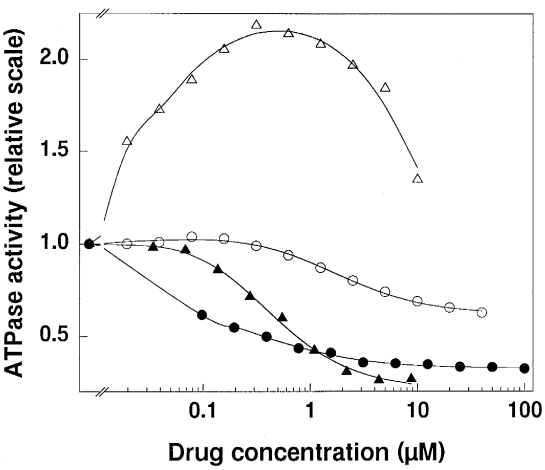

Fig. 6. Effect of hydrophobic peptides on the ATPase activity of P-glycoprotein: cyclosporin A (○), PSC-833 (●), valinomycin (△), and gramicidin S (▲).

vanadate (VO_4). For all three cases vanadate inhibits the ATPase, for the resistant lines verapamil stimulates the activity, but the CR1R12 membranes have by far the greatest, specific ATPase activity. This cell line constitutively overexpresses P-glycoprotein, which amounts to a maximum of 32% (w/w) of the total plasma membrane protein [8]. Thus, the CR1R12 cell line provides an excellent source of membranes enriched in P-glycoprotein, and enables a characterization of the P-glycoprotein associated ATPase activity. We here investigate the P-glycoprotein associated ATPase activity with respect to substrate specificity and drug modulation pattern.

3.2. Activation energy for the ATPase reaction

A typical drug-activation pattern, in this case for verapamil at four different temperatures, is depicted in Fig. 2A. The ATPase activity profile is a characteristic bell-shape, indicating the presence of two binding sites for the drug; one of high affinity, activating, and the second, low-affinity site, inhibiting the ATPase activity. As in the preceding paper the data are fitted (solid lines) to the equation:

$$V_{(S)} = \frac{K_1K_2V_0 + K_2V_1S + V_2S^2}{K_1K_2 + K_2S + S^2} \quad (1)$$

where $V_{(S)}$ is the ATPase activity as a function of the substrate concentration S, V_0 is the basal activity (the activity in the absence of modulator), V_1 is the maximal enzyme activity (if only activation occurred), and K_1 is the substrate concentration that gives half this maximal increment in ATPase activity. V_2 is the activity at infinite concentration of the modulator, and K_2 is the substrate concentration which gives half-maximal reduction of ATPase activity from the value V_1.

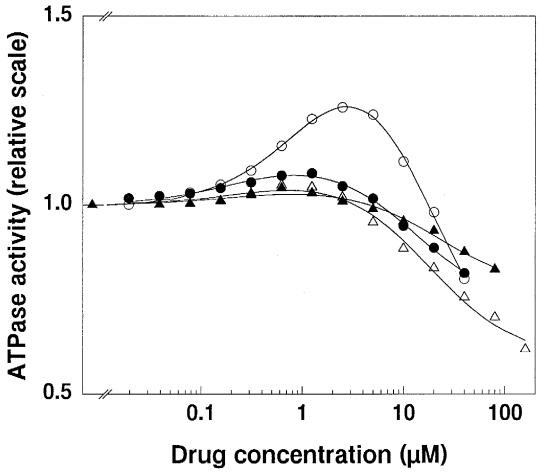

Fig. 7. Effect of vinca alkaloids and anthracyclines on the ATPase activity of P-glycoprotein: vinblastine (○), vincristine (●), daunorubicin (△), and epirubicin (▲).

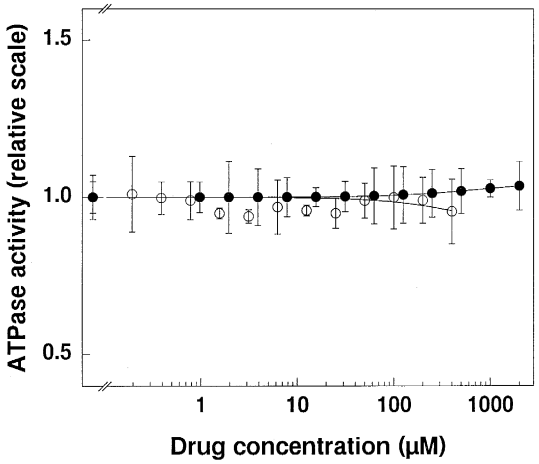

Fig. 8. Two drugs that show no apparent modulation of the P-glycoprotein ATPase: colchicine (○) and methotrexate (●).

Fig. 2B is an Arrhenius plot of the basal activity (V_0), the maximal activity (V_1), and the increase in activity ($V_1 - V_0$) as a function of the reciprocal of the absolute temperature. The lines drawn are first order regressions to the data, the slopes of which give the activation energy, which was 77.2 kJ/mol for V_1 and 72.9 kJ/mol for $V_1 - V_0$. This is the first time that data have been presented on the activation energy of the P-glycoprotein ATPase. The values found compare well with the value of 71 kJ/mol for ATP hydrolysis by the Na^+/K^+-ATPase from guinea-pig kidney [16] and with the activation energy of 70 kJ/mol for the myosin ATPase from rabbit muscles [17].

3.3. ATPase activity profiles for 34 drugs

We have characterized the ATPase activity profiles for 34 drugs. Figs. 3–8 depict these data for various classes of modulators: The calmodulin antagonists (Fig. 3), steroids (Fig. 4), hydrophobic cationic drugs (Fig. 5), hydrophobic peptides (Fig. 6), chemothera-

peutic substrates of P-glycoprotein (Fig. 7), and two drugs that show no apparent modulation of the ATPase (Fig. 8).

We found for all 34 drugs, that Eq. (1) gave a statistically better fit to the data than a simple one-site Michaelis-Menten equation. In all except one case (PSC-833) the improvement of the fit was better at $P < 0.025$, and for PSC-833 the improvement was better at $P < 0.1$. Table 1 summarizes the kinetic parameters derived from this analysis.

3.4. Structure-activity relationships

We first asked whether there was any relationship between the kinetic parameters and the partition coefficients for the various drugs. Fig. 9 depicts a plot of $1/K_1$ (Fig. 9A) and of V_1 (Fig. 9B) versus log of the octanol-water partition coefficient (P). These partition coefficients were estimated on the basis of atomic parameters derived by Ghose and Crippen [18]. The straight line in each figure is the linear regression.

Table 1
Kinetic parameters for P-glycoprotein associated ATPase activity as a function of different drugs and drug concentrations

Drug	$Y = (K_1K_2V_0 + K_2V_1S + V_2S^2)/(K_1K_2 + K_2S + S^2)$, $V_0 = 1$			
	V_1 (fold)	V_2 (fold)	K_1 (μM)	K_2 (μM)
± -Verapamil	2.1	0.6	2.5	98.5
D-verapamil	2.1	0.4	1.6	164
Dipyridamole	1.9	1.3	0.4	190
Diltiazem	3.3	0	73.3	677
Fluphenazine	2.1	0.7	11.3	28.8
Trifluoperazine	2.0	0.6	6.5	24.1
Triflupromazine	2.0	0.8	15.7	59.2
Chlorpromazine	1.8	0.6	12.2	80.9
Amiodarone	2.0	0.4	3.2	7.2
Pimozide	1.6	0.2	1.0	11.1
Propafenone	2.0	0	4.2	632
Quinidine	1.6	0	5.0	1187
Amitriptyline	2.3	0.9	108	110
Promethazine	1.8	0	23.4	1770
Progesterone	2.6	0.3	17.5	294
Spironolactone	1.9	0.4	3.9	451
Reserpine	1.3	0.02	0.1	50.9
S-Propranolol	2.1	0.9	170	551
Terfenadine	1.6	0.4	2.2	8.1
Fucidin	1.2	0.2	4.2	129
Mefloquine	2.1	0.4	3.8	9.2
Tamoxifen	1.8	0.1	0.1	11.9
Cyclosporin A	1.06	0.6	0.06	1.7
PSC-833	0.6	0.3	0.03	0.7
Valinomycin	2.3	0	0.03	16.1
Gramicidin S	1.03	0.2	0.09	0.4
Vinblastine	1.5	0.5	1.3	17.3
Vincristine	1.1	0.7	0.2	12.9
Daunorubicin	1.08	0.6	0.3	18.2
Epirubicin	1.05	0.8	0.2	22.8
Colchicine	1.08	1.08 [1]	842	1010
Methotrexate	0.9 [2]	0.9	575	4625

The relative ATPase activity of CR1R12 derived microsomes has been used for the curve fits, applying the Marquardt-Levenberg algorithm. [1] The activity did not decline, even at the highest drug concentration. [2] The activity did not change with drug concentration, hence, the fit reflects random fluctuations only.

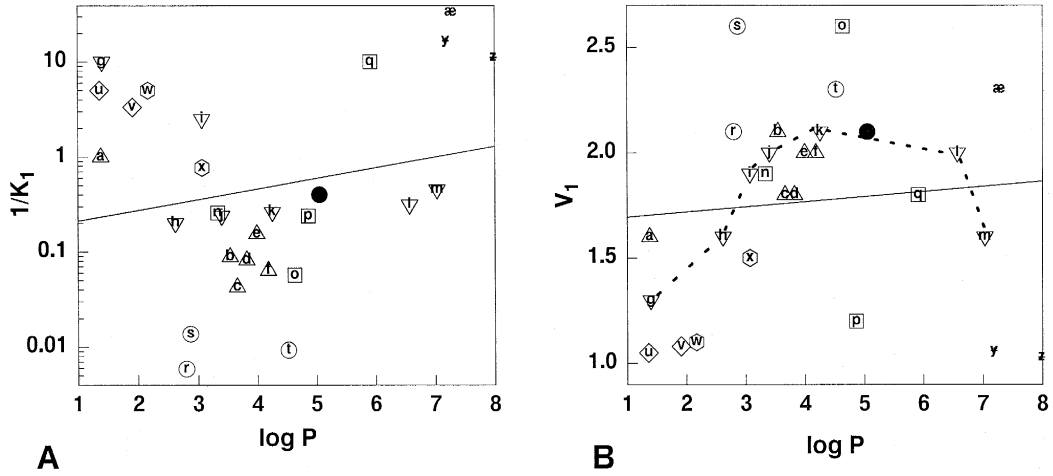

Fig. 9. Correlation between (A) $1/K_1$ for ATPase stimulation (log scale) and log P, and (B) maximal ATPase activity V_1 and log P. Symbols: **verapamil** (●); **calmodulin antagonists** (△): pimozide (a), fluphenazine (b), promethazine (c), chlorpromazine (d), trifluoperazine (e), triflupromazine (f); **hydrophobic cationic drugs** (▽): reserpine (g), quinidine (h), dipyridamole (i), propafenone (j), mefloquine (k), amiodarone (l), terfenadine (m); **steroids and tamoxifen** (□): spironolactone (n), progesterone (o), fucidin (p), tamoxifen (q); **low affinity drugs** (○): propranolol (r), diltiazem (s), amitriptyline (t); **anthracyclines** (◇): epirubicin (u), daunorubicin (v); vinca alkaloids (hexagons): vincristine (w), vinblastine (x); **hydrophobic peptides** (+): cyclosporin A (y), gramicidin S (z), valinomycin (æ). The straight lines are first order regression lines; the dotted line (B) accentuates the trend in the series of hydrophobic cationic drugs.

The correlation coefficient for $1/K_1$ on log P is 0.206, and for V_1 on log P is 0.099, neither of which is significant. However, it could be argued, that except for one point (p, fucidin), V_1 rises with log P to a maximum at log $P = 4.3$ and then falls again.

Fig. 10 depicts a similar plot of $1/K_1$ (Fig. 10A) and of V_1 (Fig. 10B) versus the Van der Waals surface area, again estimated according to the procedure of Ghose and Crippen [18]. The solid straight line is the linear regression. The correlation coefficient for $1/K_1$

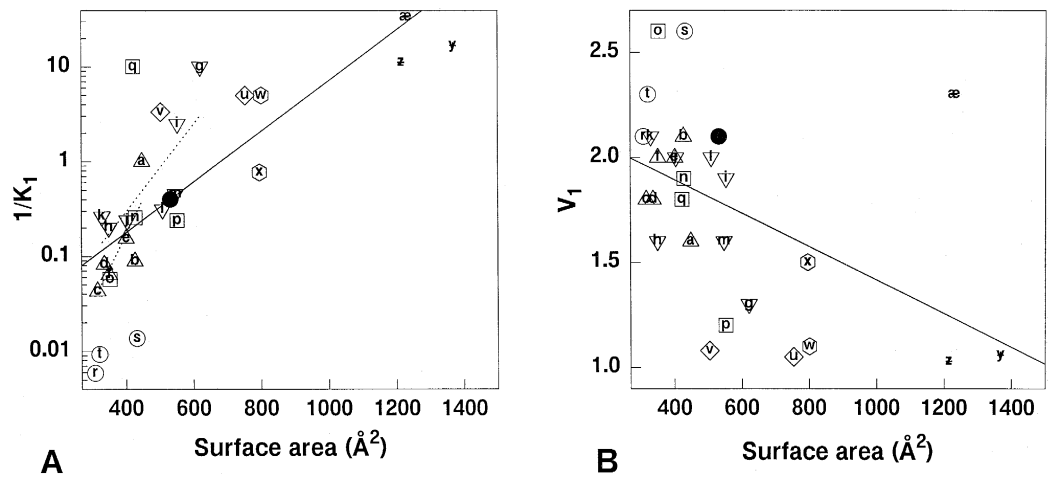

Fig. 10. Correlation between (A) $1/K_1$ (log scale) for ATPase stimulation and Van der Waals surface area, and (B) maximal ATPase activity V_1 versus Van der Waals surface area. The same symbols as in Fig. 9 apply. The straight lines are first order regressions; the dotted lines (A) are regression lines for the series of hydrophobic cationic drugs and the calmodulin antagonists.

on surface area is 0.753, which is significant at $P < 0.01$. The dotted lines are regressions through two subsets of closely related chemical structures: the phenothiazines of Fig. 3, and the hydrophobic cations of Fig. 5. In both series $1/K_1$ increases with surface area.

The plot of V_1 versus surface area is depicted in Fig. 10B. The straight line is the linear regression of negative slope with a correlation coefficient of 0.494, significant at $P < 0.01$.

4. Discussion

The mechanism by which a wide variety of structurally and physically unrelated drugs are able to interact with P-glycoprotein is essentially unknown [19]. The structure-function relationship of chemosensitizers has been put into focus, because such knowledge might lead to design of more efficient inhibitors of P-glycoprotein. However, so far, it has not been possible to identify any structural features, which were strongly correlated with the reversing capacity of the compounds [20]. Chiba et al. [3] examined a series of propafenone derivatives; no stereoselectivity was observed, a tertiary amino group enhanced the effectiveness, and the highest activity was seen with compounds having three aromatic groups and a piperazine moiety. However, none of these compounds were as potent as the reference substance, verapamil. Pearce et al. [21] reached a somewhat similar conclusion by studying a series of reserpine analogs, in which the arrangement of aromatic groups and a basic nitrogen atom determined the activity. Gros and co-workers [2] employed a series of colchicine analogs to identify structural determinants that are required for recognition by P-glycoprotein, and found that both a minimal size and a nitrogen atom at the acetamido group of colchicine are essential for efficient interaction with P-glycoprotein. In a study of the structural features of phenothiazines Ford et al. [22] emphasized the importance of a hydrophobic core for the 'ideal reverser'. Zamora et al. [1] investigated a wide range of modulators, and found that the reversing potential was best correlated to the octanol/water partition coefficients of the drugs. This finding was confirmed by Pearce et al. [4], who found the strongest reversers to be the most hydrophobic, and suggested that ''structural similarities between different classes of compounds are present in compounds recognized by the MDR phenotype'', thus emphasizing the importance of a ligand-receptor relationship for the reversers of P-glycoprotein.

While in most of the above studies a specific interaction between the modulator and some binding site on P-glycoprotein is assumed, other investigators have suggested that the chemosensitizing ability of many modulators is primarily due to their nonspecific membrane perturbing effect [23–25], like the sensitizing action of detergents [26] and surfactants [27] on MDR cells. Since P-glycoprotein is a membrane-spanning protein, one would expect its transport function to be affected by the physical properties of the membrane and of the surrounding lipid environment [28]. Therefore, the possible membrane interactions of MDR modulators should not be neglected when analyzing the mechanism of action of the drugs [23]. However, when examining the concentration dependence of resistance reversal, it appears that most of the reversers efficiently inhibit P-glycoprotein mediated transport at concentrations which are lower than those which cause membrane perturbations. For example, verapamil has a K_i of 47 μM for displacement of rhodamine 6G from phospholipids [23], while the concentrations which reverse MDR are in the range 1–10 μM [19,29,30].

The aim of this study was to discern any trends in P-glycoprotein ATPase modulating ability of a series of 34 drugs, varying in structure as well as in biological activity. To this end, we have performed a complete kinetic analysis to separate out the effects of structural variables on the affinity and maximum velocity of the ATPase (Table 1). In most cases (25 out of 34), stimulation of the P-glycoprotein associated ATPase activity developed at low drug concentrations and was describable by simple Michaelis-Menten saturation kinetics (Figs. 2–8). However, at higher substrate concentrations, marked inhibition of the ATPase activity consistently occurred (all drugs, excepting propranolol). This characteristic biphasic response strongly suggests that at least the activation of the P-glycoprotein ATPase is a specific effect due to direct interaction between the substrate and the catalytic sites of P-glycoprotein. Whether the decrement in activity observed at higher drug levels was due to a nonspecific membrane-altering effect or

whether it reflected interaction with a low-affinity inhibitory site at P-glycoprotein is at present not clear. For those drugs that elicited inhibition of ATPase activity at relatively low concentrations (K_2 around 1 μM: cyclosporin A, PSC-833, gramicidin D and S) this effect was in all probability due to specific interactions with P-glycoprotein. Most drugs, however, caused inhibition at concentrations between 10 and 1000 μM, and such high levels are likely to be associated with nonspecific perturbation of the membrane structure. This conclusion was supported by experiments with microsomes prepared from drug sensitive Ehrlich ascites cells (data not shown), the ATPase activity of which was also decreased by those drugs (the phenothiazines, amiodarone, reserpine, terfenadine, fucidin, and tamoxifen) that had a noticeable inhibitory effect on P-glycoprotein containing membranes.

The fact that a number of drugs showed ATPase activity profiles similar to those of daunorubicin and gramicidin S (Fig. 2) shows that these drugs are able to reduce the ATPase activity below the basal level. This suggests that basal activity is, at least in part, due to P-glycoprotein. This conclusion is reinforced by our finding that both the basal activity and the drug-stimulated activity are greatly enhanced in the highly P-glycoprotein expressing CR1R12 cell line.

The majority of the drugs (28 out of 34) had high affinity for the P-glycoprotein ATPase, with an apparent K_m between 0.1–10 μM, supporting the view that drug-modulated ATPase activity is reflecting specific drug-protein interactions. A few substances had relatively high K_m values, namely diltiazem (41 μM), amitriptyline (46 μM), propranolol (72 μM), and in particular colchicine (775 μM).

Our data support the findings of Pirker et al. [31] and suggest that stereospecificity is not a feature of P-glycoprotein. Thus, the kinetic parameters of ATPase activity stimulation for both the racemate and the enantiomers of verapamil were not different, and the same was tue for the R- and S-enantiomers of propranolol.

Contrary to the findings of Zamora et al. [1] and of Pearce et al. [4] we find no statistically significant dependence of either of the two kinetic parameters $1/K_1$ (Fig. 9A) or V_1 (Fig. 9B) on partition coefficient (We use $1/K_1$ rather than K_1 in Fig. 9AFig. 10A because it is the reciprocal of the Michaelis constant, Km, which is directly proportional to affinity, and we plot log $(1/K_1)$ rather than $1/K_1$, because log $(1/K_1)$ is directly proportional to the free energy of interaction between ligand and receptor). Even if we consider the three series of structurally related compounds that appear in our data set, we find no consistent variation of $1/K_1$ with log P. If, however, we look at V_1 versus log P, the data for the hydrophobic drugs (dotted line in Fig. 9B) show a clear maximum for mefloquine. This agrees well with 'Ferguson's principle' [5] which states that biological potency increases with partition coefficient until an optimum value is reached, after which a fall-off in activity is observed, probably as a result of limiting aqueous solubility.

What we do find, is a very clear correlation of the affinity of the modulators with their Van der Waals surface area. The data as a whole (solid line in Fig. 10A), and the individual series (dotted lines), show good linear dependence of log $(1/K_1)$ on the Van der Waals surface area. This suggests that binding between modulators and P-glycoprotein takes place across a wide interaction surface on the protein.

Considering now, the maximum velocity of ATPase activity, here we find an overall decrease with increasing surface area (excepting valinomycin). This would be expected if it was the de-binding of the ligand that was the rate-limiting step for the overall ATPase activity, but it would also be the case if a rate-limiting conformational change of the enzyme was impeded by a bulky substrate.

The good correlation between affinity and molecular size would seem to be an important finding, since it partly accounts for the lack of specificity of P-glycoprotein. These findings favor a model of P-glycoprotein with 'pliable' domains as suggested by Sehested [32], e.g. a large drug-binding region, rather than multiple high-affinity sites.

Acknowledgements

The authors are indebted to Dr Alan E. Senior, University of Rochester Medical Center (New York, USA) for kindly providing the CHO CR1R12 cell line. Thomas Litman is the recipient of research grants from the Danish Cancer Society (grant no. 11 94 039), the Novo Nordisk Foundation, and 'Tribute

to the Danes through Scholarships in Israel'. The authors thank Birthe Lynderup and Bente Raatz for excellent technical assistance.

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