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Effects of cardiovascular drugs on ATPase activity of P-glycoprotein in plasma membranes and in purified reconstituted form

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Abstract

Drug interactions with P-glycoprotein (Pgp) were quantitatively assessed using ATPase assay. Two experimental systems were used, (i) plasma membranes isolated from a multidrug-resistant cell line, which contained 30% Pgp as fraction of total membrane protein, and (ii) purified reconstituted Pgp. The cardioactive drugs verapamil, quinidine, diltiazem, nifedipine, and a series of digitalis analogs, interacted directly with Pgp as shown by effects on ATPase in both systems. Apparent affinities of drug binding were calculated. Direct competition was shown between digitoxin and verapamil. Drug–drug interaction in vivo at the level of Pgp is expected from the results. This approach seems well-suited for empirical determination of drug interactions with Pgp, and prediction of drug–drug interactions. © 1998 Elsevier Science B.V.

Keywords: Drug-P-glycoprotein interaction; P-glycoprotein; ATPase

1. Introduction

P-glycoprotein (Pgp) is a plasma membrane glycoprotein that confers multidrug-resistance (MDR) phenotype by virtue of its ability to exclude a wide range of drugs and other hydrophobic compounds from cells in an ATP-dependent manner. Its mechanism of action is not yet understood, the most widely-discussed current hypothesis is that it uses the energy of ATP hydrolysis to export drugs from the cell. Multidrug-resistance is an important obstacle to successful chemotherapy of human cancer, thus there is considerable interest in the role of Pgp in this phenomenon, and in devising methods to circumvent or overcome it. Recent reviews of this topic may be found in [1–5].

Amino acid sequences of Pgp from human and rodents have established that it consists of ~1280 residues, predicted to form a tertiary structure that may be described schematically as {TMD1–NBS1–TMD2–NBS2} where TMD indicates a transmembrane domain of six-helices and NBS indicates a cytoplasmic-sided nucleotide binding site. The sequence and predicted structure of Pgp characterize it as a prominent member of the family of ABC transporters [6].

Many, diverse compounds that reverse MDR in cultured cells have been identified. Called chemosensitizers or MDR modulators, many are thought to either bind directly to Pgp or act as inhibitors of drug transport via indirect biophysical effects on plasma

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membrane fluidity [7]. They include calcium channel blockers, calmodulin inhibitors, immunosuppressants, steroid hormones, the antiestrogen tamoxifen, and the opiate antagonist naloxone [8,9]. As the list of drugs which modulate MDR phenotypes continues to expand, there has been appreciation of possible interference with physiological function of naturally-expressed Pgp. In humans, Pgp is found in secretory epithelial tissues, including brush border of renal proximal tubules, canalicular membranes in liver, apical membranes lining the gut, also the adrenal gland, placental trophoblast, and endothelial blood barrier in brain and testes [10–12]. This localized expression suggested that Pgp may function normally to clear xenobiotics from blood, protect vital organs from toxic insult, and transport steroids. Experiments with genetically-engineered ''knockout'' mice have supported these ideas [13,14]. Thus, the possibility of adverse side-effects of Pgp chemosensitizers is of significant concern [14].

Several of the drugs that have been discovered to be MDR modulators are currently in clinical use. Examples are cardioactive drugs that have been used for many years to treat angina, arrhythmia, and congestive heart failure, including calcium channel blockers such as verapamil and diltiazem, antiarrhythmia agents such as quinidine, and cardiac glycosides such as digoxin and digitoxin. It has been proposed that some of the complications and adverse reactions observed upon administration of these drugs [15] may possibly be explained by interactions with Pgp, leading to inhibited drug clearance, and exacerbated drug toxicity associated with elevated plasma or tissue concentration [14,16].

Pgp in plasma membranes shows a ''basal'' rate of hydrolysis of MgATP, which is stimulated by several fold on addition of drugs, and the turnover rate is relatively respectable compared to that of ion-transporting ATPases [17–20]. A valuable approach to characterize potentially useful chemosensitizers has been to assay effects on drug-stimulated Pgp ATPase activity in plasma membranes isolated from cells which overexpress Pgp [21–23]. This approach allows the affinity of the chemosensitizer and the type of inhibition to be characterized by kinetic analysis. In this paper, we first characterize interaction of a series of cardioactive drugs with Pgp, by measuring effects on Pgp ATPase activity in plasma membranes which are isolated from a multidrug-resistant Chinese hamster ovary cell-line and are highly-enriched in Pgp. We then extend the approach by comparing the results with effects seen on ATPase activity of purified Pgp reconstituted in liposomes. The data allow quantitative assessment of interactions of cardioactive drugs with Pgp. We also characterized the effects of tamoxifen, progesterone and naloxone on Pgp ATPase activity in both plasma membranes and purified reconstituted Pgp.

2. Experimental procedures

2.1. Preparation of plasma membranes

Plasma membranes were prepared from the multidrug-resistant Chinese hamster ovary cell line CR1R12 as in [24,25]. Plasma membranes used in this study contained 30% Pgp (w/w) as a fraction of total protein, determined as described in [26]. The specific Pgp ATPase activity in absence of drug was ~ 1.25 μmol/min/mg membrane protein.

2.2. Purification and reconstitution of P-glycoprotein

Pgp was purified from plasma membranes and reconstituted in proteoliposomes as previously described [27], except that the *E. coli* lipids used previously were replaced by a mixture of lipids consisting of *E. coli* lipids, L-phosphatidylcholine (egg lecithin), phosphatidylserine (bovine brain extract), and cholesterol, in a ratio of 60/17.5/10/12.5, respectively [28]. The specific activity of pure Pgp was 3.0 μmol ATP hydrolyzed/min/mg protein in absence of added drug.

2.3. Assay of ATPase activity

ATPase activity was assayed at 37°C as described in [24]. Briefly, 5 μg of plasma membrane or 0.5 μg of pure reconstituted Pgp protein in 5 μl was added to 200 μl of ATPase assay medium containing 40 mM Tris–Cl, pH 7.4, 0.1 mM EGTA, 15 mM $MgSO_4$ and 10 mM Tris–ATP [29] at 37°C. 50 μl aliquots were quenched at appropriate times in 1 ml ice-cold 20 mM H_2SO_4 and Pi was determined [30]. Solutions of drug were made freshly from stocks in dimethylsulfoxide

(DMSO) with the final concentration of DMSO not $> 1\%$ (v/v) in the assay. Linearity of reaction was confirmed in all experiments, and ATP hydrolyzed was $< 10\%$ of total ATP. MgATP concentrations were calculated according to [31].

2.4. Statistical analysis

Data from experiments measuring ATP hydrolysis vs. [MgATP] or [verapamil] were fit to the Michaelis-Menten equation by nonlinear least squares regression analysis using Sigma Plot 4.11. All curve fits correlated with data with $P < 0.05$ as determined by paired Student's t test. V_{max} and K_m values with standard errors were derived from these curves (Table 1) and K_i values were calculated using the equation:

$$K_i = \frac{[I]}{((K_{mi}/K_{mo}) - 1)}.$$

2.5. Routine procedures

SDS gel electrophoresis and protein assay by bicinchoninic acid method in the presence of 1% SDS were performed as previously described [24].

Table 1
Characteristics of P-glycoprotein derived from ATPase assay

	Plasma membranes	Purified Pgp
K_m(MgATP) (mM)		
no drug	1.4 (0.3)	0.8 (0.11)
10 μM digitoxin	1.9 (0.5)	0.9 (0.15)
30 μM digitoxin	1.9 (0.5)	0.7 (0.08)
V_{max} (μmol/min/mg)		
no drug	0.81 (0.06)	3.4 (0.16)
10 μM digitoxin	0.67 (0.07)	2.7 (0.16)
30 μM digitoxin	0.56 (0.06)	2.0 (0.08)
K_m(verapamil) (μM)		
no drug	0.33 (0.02)	0.4 (0.13)
3 μM digitoxin	0.61 (0.08)	0.7 (0.25)
10 μM digitoxin	1.40 (0.35)	1.4 (0.36)
V_{max} (μmol/min/mg)		
no drug	2.8 (0.88)	4.9 (0.15)
3 μM digitoxin	2.6 (0.71)	4.4 (0.13)
10 μM digitoxin	2.7 (0.56)	4.3 (0.21)
K_i(digitoxin) (μM)	3.5	3.5

The parameters with standard error in parentheses are derived from the experiments in Figs. 3 and 4.

For protein assay, proteoliposomes were dilapidated by the method of Wessel and Flugge [32].

2.6. Materials

Acetone/ether precipitated *E. coli* lipids and cholesterol were obtained from Avanti Polar Lipids; L-phosphatidylcholine from egg yolk and phosphatidylserine from bovine brain extract (Folch fraction III) were obtained from Sigma. Dye-ligand chromatography reagent Reactive Red 120 agarose Type 3000-Cl, routine chemicals, and drugs, were from Sigma. Octylglucoside (OG, *n*-octyl-D-glucopyranoside) used in the solubilization and purification of Pgp was from Calbiochem.

3. Results

3.1. P-glycoprotein in plasma membranes and purified reconstituted form

In this work, we used two preparations to assay effects of drugs on Pgp ATPase activity. The first was a plasma membrane preparation from the multidrug-resistant Chinese hamster ovary cell line CR1R12, which constitutively overexpresses Pgp [24]. The plasma membranes contained 30% Pgp as percentage of total membrane protein. Na^+, K^+-ATPase activity could be eliminated either by inclusion of ouabain (2 mM), or by exclusion of Na^+ and K^+ from the assay. Here we used the latter approach. The ATPase activity of Pgp with ouabain and physiological concentrations of Na^+ and K^+ present in the assay medium was not significantly different from that measured without Na^+ and K^+ (data not shown). Ca^{2+}-ATPase, which was very low, was eliminated by inclusion of EGTA, and ecto-ATPase and mitochondrial ATPase were absent. The second preparation was purified Pgp reconstituted in liposomes. During purification of Pgp we substituted here a lipid mixture more similar to that of mammalian plasma membranes for the *E. coli* lipids used previously [27], to achieve a more native environment. The purity of the Pgp was equal to that seen previously [27]. Pure Pgp showed no Na^+, K^+-ATPase activity. In both plasma membranes and purified reconstituted Pgp, ATPase activity was completely inhibited by 100 μM vanadate.

3.2. Effects of calcium-channel blockers and quinidine on ATPase activity

Pgp ATPase activity was stimulated by verapamil, diltiazem, nifedipine, and quinidine. Fig. 1(A) and (B) show the concentration-dependent effects of these drugs in plasma membranes and purified reconstituted Pgp, respectively. Verapamil and quinidine stimulated maximally at 10 μM, nifedipine at 30 μM and diltiazem at 100 μM. Concentration required for half-maximal stimulation was 1 μM (verapamil, quinidine) or 3 μM (nifedipine, diltiazem). All four drugs displayed a biphasic effect; as concentration increased beyond that which stimulated maximally, activity then decreased. As shown in Fig. 1(B), verapamil, quinidine and diltiazem also stimulated ATPase activity of pure reconstituted Pgp, the effects resembling those measured in plasma membranes. Results with nifedipine are not included in Fig. 1(B), since, only one experiment was conducted, but the pattern observed was closely similar to that seen in Fig. 1(A). The data show that these cardioactive drugs all interact with Pgp directly, and establish the concentrations at which in vivo effects are likely.

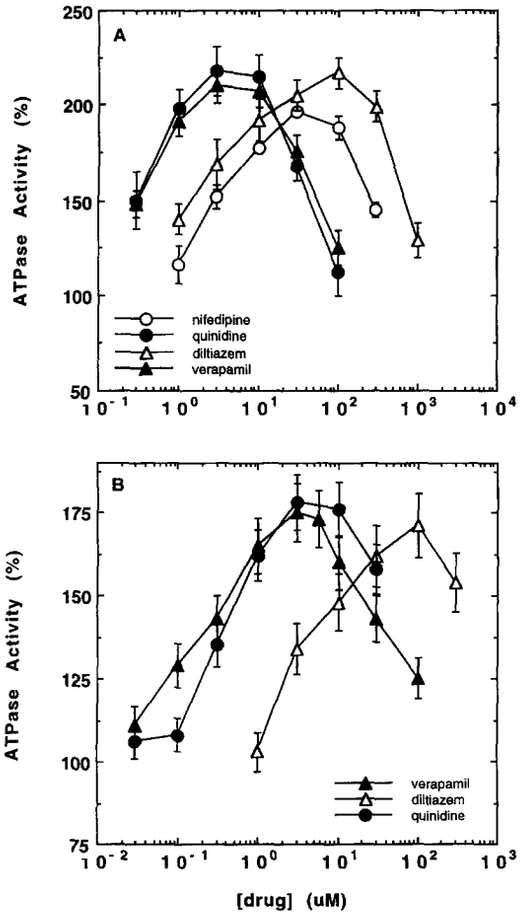

Fig. 1. Effects of calcium channel blockers and quinidine on ATPase activity of P-glycoprotein. (A) Plasma membranes, (B) Purified reconstituted Pgp. ●, quinidine; ▲, verapamil; ○, nifedipine; △, diltiazem. Each data point represents the mean of at least three experiments, ± SEM. 100% activity in each case refers to ATPase measured with no drug addition.

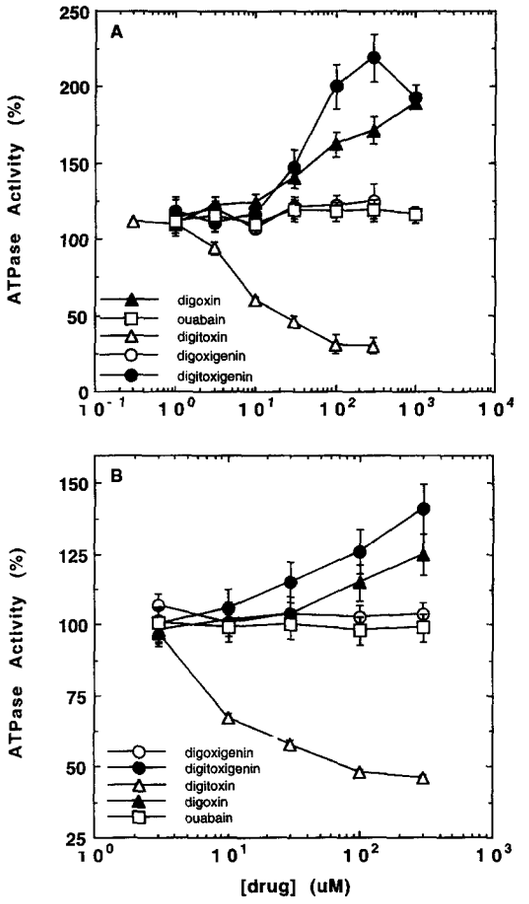

Fig. 2. Effects of cardiac glycosides on ATPase activity of P-glycoprotein. (A) Plasma membranes, (B) Purified reconstituted Pgp. ●, digitoxigenin; ▲, digoxin; ○, digoxigenin; △, digitoxin; □, ouabain. Each data point represents the mean of at least three experiments, ± SEM. 100% activity in each case refers to ATPase measured with no drug addition.

3.3. Modulation of Pgp ATPase activity by cardiac glycosides

Cardioactive steroids and glycosides displayed remarkably diverse effects on Pgp, and as Fig. 2(A)

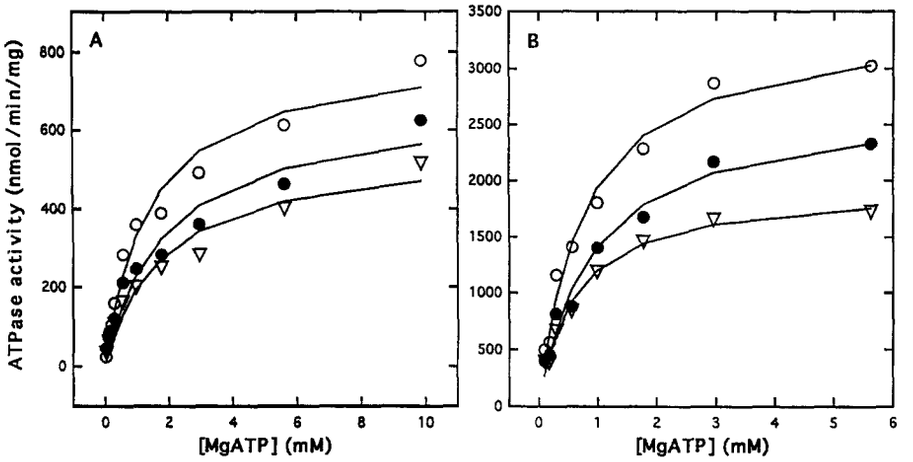

Fig. 3. Dependence of ATPase activity of P-glycoprotein on MgATP concentration in the presence and absence of digitoxin. (A) Plasma membranes, (B) Purified reconstituted Pgp. K_m(MgATP) and V_{max} values were determined by fitting data to the Michaelis-Menten equation as described in Section 2. ○, no digitoxin; ●, 10 μM digitoxin; ▽, 30 μM digitoxin. Each data point represents the mean of at least three experiments, which shared excellent agreement.

and (B) show, the effects were seen consistently in both plasma membranes and pure reconstituted Pgp. Both the aglycone digitoxigenin and the glycoside digoxin stimulated Pgp ATPase activity in a concentration-dependent manner. Digoxigenin and ouabain had neither stimulatory nor inhibitory effect, while digitoxin inhibited ATPase activity. These data established that cardiac glycosides interact directly with Pgp.

We examined whether cardiac glycosides affected K_m(MgATP) by measuring ATPase activity as a function of MgATP concentration in presence and absence of fixed concentrations of digitoxin. The data are shown in Fig. 3(A) and (B), and the calculated K_m(MgATP) and V_{max} values from nonlinear least squares fits to the Michaelis-Menten equation are given in Table 1, upper half. In both plasma membranes and pure Pgp, V_{max} was reduced by 10 and

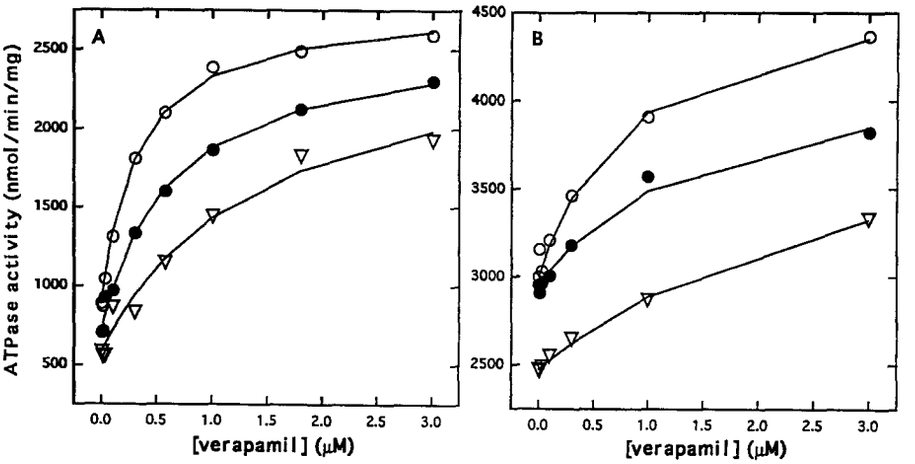

Fig. 4. Verapamil-stimulation of P-glycoprotein ATPase activity measured in the presence of digitoxin. (A) Plasma membranes, (B) Purified reconstituted Pgp. K_m(verapamil), K_i(digitoxin) and V_{max} values were determined by fitting data to the Michaelis-Menten equation as described in Section 2 and are given in Table 1. ○, no digitoxin; ●, 3 μM digitoxin; ▽, 10 μM digitoxin. Each data point represents the mean of at least three experiments, which shared excellent agreement.

30 μM digitoxin, but apparent K_m(MgATP) was essentially unchanged. These data indicated that digitoxin did not interfere with binding of MgATP. A similar set of experiments with digitoxigenin yielded the same conclusion (data not shown). It seemed likely, therefore, that the cardiac glycosides were acting by binding to the drug-binding site(s) in the Pgp membrane domains. This was examined by assaying ATPase activity in presence of concentrations of verapamil which were shown to stimulate Pgp with zero, 3 or 10 μM digitoxin also present. The data are shown in Fig. 4(A) and (B). Fitting these data by nonlinear least squares regression analysis, assuming simple Michaelis-Menten kinetics, revealed that the apparent K_m(verapamil) was 0.33 μM in plasma membranes and 0.4 μM in purified reconstituted Pgp (Table 1, lower half). In presence of digitoxin, apparent K_m(verapamil) was increased but no major changes in V_{max} values occurred, suggesting that digitoxin competes for binding with verapamil. K_i(digitoxin) was calculated to be 3.5 μM in both plasma membranes and purified reconstituted Pgp.

Our results suggest strongly that cardiac glycosides interact with Pgp directly at the site(s) which binds verapamil, and provide quantitative assessment of the apparent affinity of the interaction. Results obtained were similar in both plasma membrane and purified, reconstituted Pgp.

3.4. Effects of tamoxifen, progesterone and naloxone on Pgp ATPase activity

We compared the effects of the anti-estrogen anticancer drug tamoxifen, the morphine antagonist naloxone, and the natural steroid progesterone, on Pgp in plasma membranes (Fig. 5(A)) and purified, reconstituted form (Fig. 5(B)). All three compounds stimulated ATPase activity in both systems. Progesterone showed maximal stimulation at the relatively high concentration of 100 μM, naloxone stimulated only at very high concentrations (> 100 μM). Results with tamoxifen were dependent upon the Pgp preparation. In plasma membranes, tamoxifen stimulated at lower (~ 10 μM) concentration and inhibited at higher (> 30 μM) concentration. With pure Pgp, degree of stimulation was greater, but occurred at higher concentration (100 μM). The data support previous results seen with plasma membranes and indicate that each of these drugs does interact with Pgp directly.

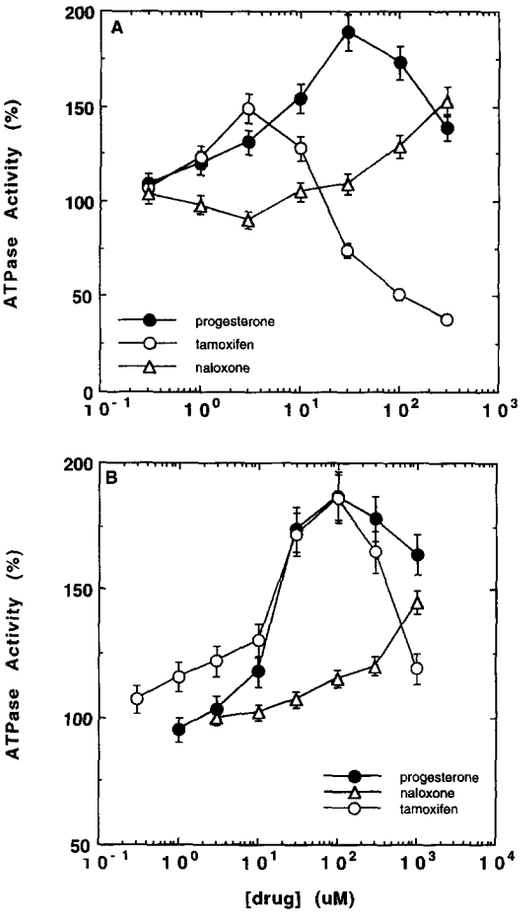

Fig. 5. Effects of tamoxifen, progesterone and naloxone on P-glycoprotein ATPase activity. (A) Plasma membranes, (B) Purified reconstituted Pgp. ○, tamoxifen; ●, progesterone; △, naloxone. Each data point represents the mean of at least three experiments, ± SEM. 100% activity in each case refers to ATPase measured with no drug addition.

4. Discussion

The goal of this study was to demonstrate and characterize the interaction of a number of drugs with membrane-bound and pure Pgp, using a sensitive in vitro ATPase assay. Two types of experimental material were used, namely plasma membranes isolated from the CR1R12 Chinese hamster ovary cell line, which constitutively overexpresses Pgp, and purified

Pgp reconstituted in liposomes using a lipid mixture similar to that of mammalian plasma membranes. Small amounts of material were sufficient for assays, and the use of purified Pgp suggests that effects seen are due to direct interaction of the drugs with Pgp. In Figs. 1–4 and Table 1 we established that a series of drugs used to treat cardiovascular disease, along with several other structurally and pharmacologically relevant drugs (Fig. 5) interact at the level Pgp, and we characterized their kinetic interaction. These kinetic parameters were used to provide indirect evidence of Pgp's apparent affinity for each of these drugs. The two Pgp preparations described here are shown to be well-suited for this purpose, and this is the first report of such application of purified Pgp. To confirm that these apparent affinities represent direct binding of substrate to Pgp would require employing equilibrium binding assays.

Binding of transport substrates or modulators by Pgp is known to be remarkably permissive, with a wide range of cationic or uncharged molecules, of diverse structure, being effective [8,9]. Subtle changes in structure may have dramatic effects on substrate efficacy, as was indicated in this work for a series of related digitalis analogs (Fig. 2). Previous comparisons of structural features of cardiac glycosides [33,34], and progesterone have not revealed molecular characteristics that correlate with observed effects on Pgp ATPase activity. The presence of the sugar moiety (Fig. 6) did not seem to be essential, as digitoxigenin and progesterone, which lack the rhamnose or digitose sugar at position C3, both stimulated Pgp. Likewise, the presence and position of a lactone ring at C17 may not be directly related to Pgp action. The degree of lipophilicity and hydroxylation of the steroid body did correlate with efficacy, with progesterone and digitoxin being most lipophilic and least hydroxylated, while ouabain and digitoxigenin are the most water soluble. In our view, the conclusion to be drawn is that, currently, empirical determination is required of whether, and in what manner, a drug interacts with Pgp.

Adverse drug reactions due to intoxication by cardiac glycosides have long been recognized [15]. Increased digoxin plasma concentrations and reduced digoxin excretion have been observed in patients upon coadministration of verapamil, diltiazem, nifedipine, or quinidine [15]. Therapeutic plasma con-

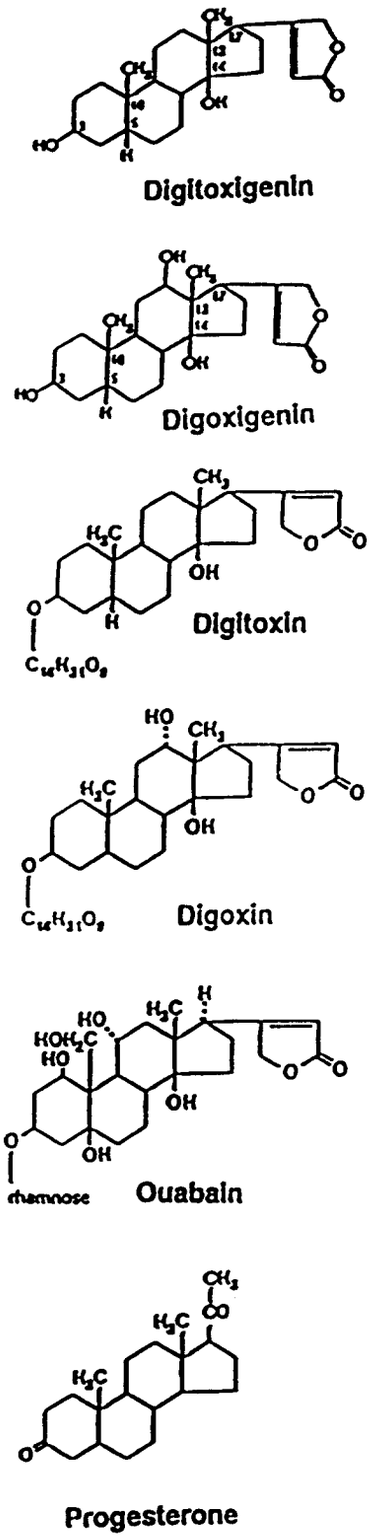

Fig. 6. Molecular structures of digitalis analogs and progesterone.

centrations of quinidine, verapamil, diltiazem, and nifedipine are typically 10 μM [35], 0.5 μM [36], 0.3 μM [37], and 0.1 μM [38], respectively. For quinidine and verapamil, these values are higher than, or similar to, the concentrations required for half-maximal saturation of Pgp (Figs. 1 and 4, Table 1). For diltiazem and nifedipine, these values are below the concentration required for half-maximal saturation of Pgp. Therapeutic plasma concentrations for digoxin and digitoxin are < 3.5 and 38 nM, respectively [39], values which are well below the apparent K_i or K_m values toward Pgp (Fig. 2, Table 1). Moreover, it was indicated that digoxin and verapamil competed for binding to Pgp (Fig. 4, Table 1). Thus the simultaneous affects of more than one drug in vivo at the level of Pgp, i.e., drug–drug interactions, are to be predicted from the quantitative parameters determined here, notably for verapamil and quinidine with digoxin or digitoxin.

Pgp is localized in luminal brush border membrane of kidney proximal tubule [10–12,40]. Firm evidence for active secretion of digoxin by net vectoral transepithelial transport has come from reports using kidney epithelial cells [14,16,41–43]. Several of these show interference in this process by verapamil or quinidine, and point to failure to excrete digoxin as one likely mode of digoxin toxicity. It has also been shown that excretion of digoxin via Pgp in intestinal mucosa is a substantial route in mice [44]. The use of genetically-engineered mice, in which one of the genes encoding Pgp is disrupted, has clearly emphasized the role of Pgp in elimination and prevention of tissue accumulation of digoxin. After administration of digoxin, elimination was notably slower in the "knockout" mice, leading to increased levels in plasma, marked accumulation in many tissues, and a truly striking accumulation in brain [13,14,44]. As the authors note, this points to possible increased risk of CNS toxicity in patients exposed to co-administration of Pgp modulators with digoxin.

Previously, morphine and other opiate agonists have been characterized as Pgp substrates. They were shown to bind specifically to plasma membranes from MDR cells, and binding was inhibited by verapamil and vinblastine in concentration-dependent manner. Naloxone, an opiate antagonist, partly inhibited vinblastine binding at concentrations of 100 μM [45]. Our results (Fig. 5) showed that naloxone, at concentrations of 100 μM and above, stimulated ATPase activity in both plasma membranes and pure Pgp, suggesting that it interacts directly with Pgp, albeit with low affinity.

Both tamoxifen and progesterone have been shown previously to be modulators of Pgp in cells and to stimulate Pgp ATPase activity in plasma membranes [21,46]. Data presented here confirm this work, and extend it to establish a direct effect also on purified reconstituted Pgp. Progesterone showed the same concentration dependence for biphasic stimulation and then inhibition of ATPase in both plasma membranes and purified reconstituted Pgp (Fig. 5). However, the concentration-dependence of the response to tamoxifen was different in the two preparations. The most likely explanation is that stimulation of ATPase activity by tamoxifen is particularly sensitive to the lipid environment of Pgp, which is known to affect drug-stimulation of Pgp ATPase activity [47]. Thus modulation of Pgp by tamoxifen in vivo may show tissue-specific characteristics.

In summary, plasma membranes from a highly MDR cell line, and purified reconstituted Pgp, provided a sensitive in vitro enzymic assay system for quantitative characterization of interaction of transport substrates and modulators with Pgp. The results demonstrated drug–drug interactions at the level of Pgp, enabling prediction of adverse reactions. Data with a group of widely-used cardiovascular drugs provide a good example of the usefulness of the approach.

Acknowledgements

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