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THE MDR1 GENE PRODUCT, P-GLYCOPROTEIN, MEDIATES THE TRANSPORT OF THE CARDIAC GLYCOSIDE, DIGOXIN

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SUMMARY: Digoxin, a widely used cardiac glycoside with a low therapeutic index, is known to interact with a large and diverse group of co-administered drugs, frequently leading to toxic accumulation of the glycoside. Establishing the mechanism(s) of these interactions, therefore, has potential clinical significance. The present studies implicate P-glycoprotein, the MDR1 gene product overexpressed in multidrug resistant cells, as the apical membrane protein responsible for the renal secretion of digoxin and provide an explanation for the occurrence of digoxin toxicity in the presence of certain co-administered medications. Since digoxin is considered a prototype for endogenous digitalis-like glycosides, the results also allow for speculation that endogenous digitalis-like glycosides may be the natural substrates for P-gp.

Digoxin is one of the most widely used drugs in the treatment of heart failure and cardiac arrythmias and, because it has a low therapeutic index, it has been associated with toxicity in infants and adults. A relatively large and diverse group of co-administered drugs are reported to interact with digoxin, for example, quinidine (1-3), verapamil (4), amiodarone (5), spironolactone (6,7) and cyclosporin (8), frequently leading to its toxic accumulation. Digoxin is eliminated to a large extent by net tubular secretion in the kidneys. Many of these drugs have been shown to decrease the renal clearance of digoxin, however, the mechanism of the renal tubular secretion of digoxin has remained largely unknown and the nephron site at which this occurs in mammalian kidney has not been established. Establishing the molecular basis for the renal secretion of digoxin could have potential clinical significance for controlling digoxin toxicity and understanding the complex pharmacokinetic interactions with other co-administered drugs.

Recently we have shown that quinidine and cyclosporin inhibit the renal secretion of digoxin across the luminal membrane of the dog kidney *in vivo* (9). Both cyclosporin and quinidine are modulators of P-glycoprotein, the MDR1 gene product overexpressed in multidrug resistant cells (10). P-glycoprotein is believed to function as an energy dependent drug efflux pump which recognizes a wide variety of cytotoxic agents. It has

Abbreviations: α-MEM, alpha minimum essential medium; CHO, chinese hamster ovary.

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also been shown to be present in relatively large amounts in many mammalian secretory epithelia, including the apical membrane of the mammalian kidney (11-13). We therefore examined the possibility that renal digoxin secretion is mediated by P-glycoprotein.

METHODS: Parent (AuxB1) and multidrug-resistant (CH^RC5) chinese hamster ovary (CHO) cells originally selected from the parent line for resistance to colchicine were grown in alpha minimal essential medium (α-MEM), containing 10% fetal bovine serum and 0.5% penicillin-streptomycin (GIBCO). Drug accumulation was determined in confluent cell monolayers on 6-well plates (Corning) subcultured 2-3 days prior. Cells were pre-incubated for 0.5 h in 1 ml of serum free α-MEM buffered with 30 mM HEPES.

To compare the accumulation of [^{3}H]vinblastine sulphate and [^{3}H]digoxin in AuxB1 *versus* CH^RC5 cells over time, drug accumulation was initiated by the addition of 0.5 ml α-MEM/30 mM HEPES containing 30 nM [^{3}H]digoxin (New England Nuclear) or [^{3}H]vinblastine sulfate (Amersham) (sp. ac. 20.5 and 8.3 Ci/mmol, respectively) and [^{14}C]inulin (extracellular marker); plates were then incubated for 0 to 4 h at 37°C in a 5% CO_2 atmosphere. Duplicate samples of each cell line were taken for each time point. Media was rapidly aspirated to stop accumulation and each well subsequently washed twice with 1 ml ice-cold 0.9% NaCl. The cells were lysed with 1N NaOH; after removal of the cells/NaOH, the wells were rinsed with 1N HCl and the cells/NaOH neutralized. After the addition of scintillation cocktail (Beckman), samples were stored in the dark overnight and the radioactivity in all samples was counted in a liquid scintillation counter (Model LS1701; Beckman) with appropriate cross-over standards. Aliquots (100 μl) of the incubation media were similarly counted. Drug binding to the wells was similarly determined by adding 0.5 ml incubation medium to wells (prepared identically to those with cells) in the absence of cells. Protein determinations (14) were performed in triplicate for each cell line; wells were incubated in parallel and processed in an identical manner. The [^{3}H]substrate measured in each sample was corrected for the corresponding fractional recovery of [^{14}C]inulin; [^{14}C]inulin measured in each sample accounted for less than 0.3% of the amount added. The amount of [^{3}H]substrate measured in each sample was also corrected for the amount of substrate bound to the well in the absence of cells.

Experiments were performed in a similar manner for comparing [^{3}H]vinblastine sulphate and [^{3}H]digoxin accumulation AuxB1 and CH^RC5 cells over time in the presence and absence of cyclosporin A. For drug accumulation measured in the presence of cyclosporin, cyclosporin A (Sandimmune$^{i.v.}$; Sandoz) was added to half of the incubation medium (α-MEM/30 mM HEPES) containing 22 and 30 nM [^{3}H]vinblastine and [^{3}H]digoxin, respectively, to a final concentration of 50 μM. Addition of cyclosporin did not change the concentration of [^{3}H]vinblastine or [^{3}H]digoxin in the incubation medium.

To study the effect of chemosensitizers on the accumulation of [^{3}H]digoxin in CH^RC5 and AuxB1 cells, incubations in the absence and presence of the chemosensitizers proceeded for 1 hour at 37°C. To aliquots of incubation medium containing 30 nM [^{3}H]digoxin and [^{14}C]inulin was added equal volumes of the various inhibitors diluted in 0.9% NaCl (cyclosporin, verapamil and quinidine) or DMSO (vinblastine) to the final concentrations indicated in Table 1. Two controls were performed for each cell line with the addition of the same volumes of either 0.9% NaCl or DMSO to the incubation medium in the absence of inhibitors. For each experiment, all samples were performed in triplicate.

RESULTS AND DISCUSSION: To investigate whether the transport of digoxin is mediated by P-glycoprotein, the accumulation of [^{3}H]vinblastine sulfate, a known substrate of P-glycoprotein, and [^{3}H]digoxin in multidrug resistant CHO cells (CH^RC5) and drug sensitive parent (AuxB1) cells was measured in cell culture as a function of time. The multidrug-resistant CH^RC5 cells were originally selected from the parent line for resistance to colchicine (180x resistant to colchicine relative to AuxB1, 30x to vinblastine (15)). Accumulation of [^{3}H]vinblastine sulfate reached equilibrium within one hour (Fig. 1A) in

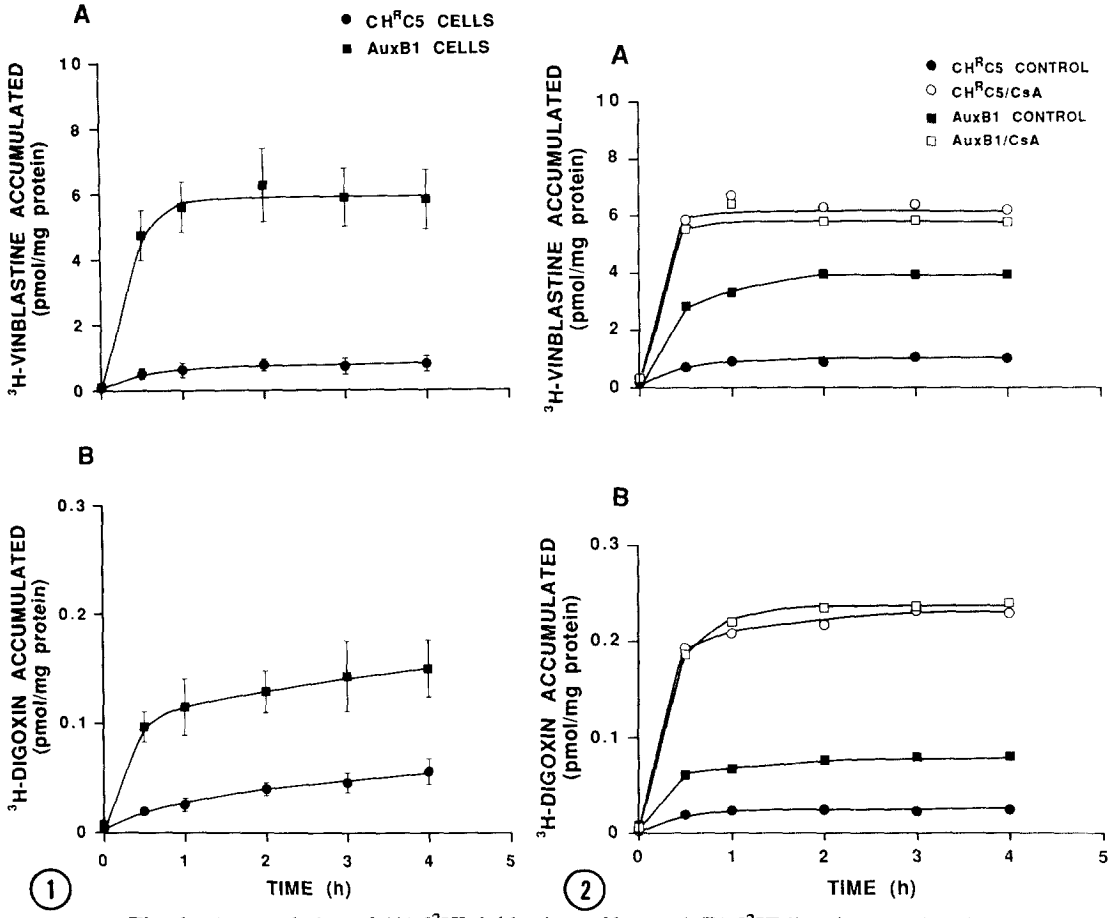

Fig. 1. Accumulation of (A) [^{3}H]vinblastine sulfate and (B) [^{3}H]digoxin over time in parental CHO AuxB1 and colchicine resistant CH^{R}C5 cells. Amounts of vinblastine and digoxin accumulated (pmol/mg protein) represent the mean±S.E. of 4 experiments.

Fig. 2. Accumulation of (A) [^{3}H]vinblastine sulfate and (B) [^{3}H]digoxin over time in drug sensitive (AuxB1) and colchicine resistant (CH^{R}C5) CHO cells in the absence (control) and presence of 50 μM cyclosporin A (CsA). One of 4 representative experiments is shown.

both cell lines. The 7 to 8-fold difference in the accumulation of [^{3}H]vinblastine sulfate in AuxB1 *versus* CH^{R}C5 cells confirms previous reports on reduced vinblastine accumulation in multidrug resistant cells, due to the extrusion of vinblastine by P-glycoprotein from the cell (15,16). Similarly, accumulation of [^{3}H]digoxin was 3 to 5-fold greater in the AuxB1 cells than in CH^{R}C5 cells, suggesting that transport of digoxin, like vinblastine, is mediated by P-glycoprotein (Fig. 1B). For both CH^{R}C5 and AuxB1 cells, accumulation of [^{3}H]digoxin over time did not reach equilibrium as rapidly, was biphasic, and was about one-tenth that of ^{3}H-vinblastine sulfate at comparable incubation medium concentrations, presumably reflecting differences in the partitioning and binding of vinblastine and digoxin into the cell and/or cell membrane. The greater cellular accumulation ratio (AuxB1/CH^{R}C5 cells) of [^{3}H]vinblastine sulfate compared to that of [^{3}H]digoxin suggests that the affinity of vinblastine for P-glycoprotein is greater than that of digoxin. When cyclosporin A (50

μM), a potent inhibitor of P-glycoprotein, was added to the incubation medium, accumulation of [^{3}H]vinblastine sulfate and [^{3}H]digoxin in the multidrug resistant CH^{R}C5 cells increased dramatically (Fig.2). The accumulation of [^{3}H]vinblastine sulfate and [^{3}H]digoxin in drug sensitive AuxB1 cells also increased when cyclosporin was present, consistent with the presence of small amounts of P-glycoprotein in these cells (15, 17). However, this latter increase was only 1.5- and 3-fold greater for [^{3}H]vinblastine sulfate and [^{3}H]digoxin, respectively, whereas the magnitude of the increase was much larger (6- and 10-fold greater for [^{3}H]vinblastine sulfate and [^{3}H]digoxin, respectively) for CH^{R}C5 cells. In the presence of 50 μM cyclosporin, when presumably P-glycoprotein mediated efflux of [^{3}H]vinblastine sulfate and [^{3}H]digoxin is completely inhibited (see below, Table 1), the accumulation of both drugs is essentially identical in CH^{R}C5 and AuxB1 cells (Fig. 2).

Two other modulators of P-glycoprotein function (verapamil and quinidine) and one substrate (vinblastine) were then investigated for their ability to inhibit/compete for

Table 1. Effect of chemosensitizers on the accumulation of [^{3}H]digoxin in CH^{R}C5 and AuxB1 cells. The data represent the mean±S.E. (n=3).

Chemosensitizer	Concentration (μM)	Cell line	[^{3}H]Digoxin accumulated (pmol/mg protein)	-Fold reversal
Control	0	CH^{R}C5	0.014±0.006	
Cyclosporin	1	CH^{R}C5	0.202±0.013	14.4
	5		0.235±0.028	16.8
	10		0.252±0.028	18.0
	50		0.257±0.025	18.4
	100		0.258±0.015	18.4
Verapamil	10	CH^{R}C5	0.034±0.008	2.4
	50		0.144±0.027	10.3
	100		0.176±0.038	12.6
Quinidine	10	CH^{R}C5	0.061±0.008	5.1
	50		0.218±0.023	18.2
	100		0.230±0.013	19.2
Control	0	CH^{R}C5	0.011+0.005	
Vinblastine	10	CH^{R}C5	0.044±0.013	4.0
	50		0.147±0.028	13.4
	100		0.157±0.039	14.3
Control	0	Aux B1	0.062±0.006	
Cyclosporin	1	Aux B1	0.256±0.048	4.1
	5		0.251±0.020	4.0
	10		0.266±0.028	4.3
	50		0.245±0.014	4.0
	100		0.231±0.036	3.7
Verapamil	10	Aux B1	0.181±0.040	2.9
	50		0.197±0.043	3.2
	100		0.193±0.048	3.1
Quinidine	10	Aux B1	0.253±0.018	3.8
	50		0.254±0.010	4.2
	100		0.228±0.013	3.7
Control	0	Aux B1	0.053±0.008	
Vinblastine	10	Aux B1	0.184±0.033	3.5
	50		0.169±0.035	3.2
	100		0.167±0.042	3.2

[^{3}H]digoxin transport. Cyclosporin, verapamil, quinidine and vinblastine increased the accumulation of [^{3}H]digoxin in CH^{R}C5 cells several-fold and in a dose-dependent manner (Table 1). These modulators/substrates also increased the accumulation of [^{3}H]digoxin in the drug sensitive AuxB1 cells, but the magnitiude of the increase was much less than that observed in the multidrug resistant cells and did not demonstrate dose-dependence. Cyclosporin was the most potent inhibitor of [^{3}H]digoxin transport; concentrations as low as 1 μM were able to increase [^{3}H]digoxin accumulation in CH^{R}C5 cells 14.4-fold (Table 1). The relative order of potency was: cyclosporin > quinidine, vinblastine > verapamil. Although vinblastine readily increased [^{3}H]digoxin accumulation (Table1), digoxin over the concentration range of 0.1 - 100 μM did not alter [^{3}H]vinblastine sulfate accumulation in either the multidrug resistant or drug sensitive cell lines (data not shown). Consistent with these results are data previously obtained in our laboratory showing that digoxin (0.6 to 100 μM) does not compete with [^{3}H]azidopine photolabelling of P-glycoprotein in rat brush border membranes or membranes prepared from CH^{R}C5 cells (9). Similar observations have been reported for other modulators/substrates (18,19), although the reasons for this remain poorly understood. However, digoxin at concentrations of 100 μM, increased [^{3}H]digoxin accumulation 4-fold in CH^{R}C5 cells versus 1.8-fold in AuxB1 cells (data not shown). The poor solubility of digoxin in aqueous medium and hence the need to dissolve digoxin in ethanol restricted the testing of higher digoxin concentrations.

Consistent with our previous studies on the excretion of [^{3}H]digoxin by the dog kidney *in vivo* (9), where cyclosporin and quinidine were found to be potent inhibitors of digoxin secretion at the luminal surface of the renal tubular cell, the present studies implicate P-glycoprotein as the apical membrane protein responsible for the renal secretion of digoxin. Further support is provided by data which show that cyclosporin A, verapamil, quinidine and vincristine inhibit the basolateral to apical flux of digoxin across LLC-PK$_1$ cell monolayers (20), which also express P-glycoprotein (21).

We conclude that P-glycoprotein mediates the cellular transport of digoxin and that the apical localization of renal tubular P-glycoprotein is responsible for the renal secretion of digoxin. Our findings have several potentially interesting implications. First, the results are relevent to the clinical pharmacology of digoxin. For example, the occurrence of digoxin toxicity in the presence of certain co-administered medications may now be explained by competition for or inhibition of digoxin transport by P-glycoprotein. In this regard, cyclosporin, quinidine and verapamil, shown here to enhance the accumulation of [^{3}H]digoxin in multidrug resistant cells (Table 1), have each been reported to interact with digoxin clinically, resulting in toxic accumulation of the glycoside (1-4,8). Amiodarone and nifedipine, two other drugs reported to interact with digoxin (5,22), are also known modulators of P-glycoprotein and hence may have a similar mechanism of interaction. In the context of tubular epithelial transport processes, the present results emphasize for the first time the potentially significant role for P-glycoprotein as a general renal drug secretory mechanism.

Finally, in a more speculative vein, we suggest that the renal secretory mechanism for digoxin mediated by P-glycoprotein represents a transepithelial transport pathway for

endogenous digitalis-like compounds. Interestingly, progesterone, a circulating steroid with digitalis-like immunoreactivity and the ability to bind to Na^+K^+ATPase (23-25), is known to interact with P-glycoprotein (26). As Na^+K^+ATPase inhibitors, such endogenous compounds can be viewed as naturally occuring and toxic, and as such would be logical substrates for P-glycoprotein. Recently, a hydrophobic extract of urine has been shown to possess remarkable potency in reversal of multidrug resistance and inhibition of [^{3}H]azidopine binding to renal brush border membranes (27). It will be of considerable interest to assay the urine extract for digitalis-like biological activity.

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