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Transport and epithelial secretion of the cardiac glycoside, digoxin, by human intestinal epithelial (Caco-2) cells

Megan E. Cavet, *Mike West & [1]Nicholas L. Simmons

Gastrointestinal Drug Delivery Research Centre, Department of Physiological Sciences, University of Newcastle upon Tyne, Medical School, Newcastle upon Tyne, NE2 4HH and *Glaxo Wellcome Medicines Research Centre, Respiratory Diseases Unit, Gunnels Wood Road, Stevenage, Hertfordshire, SG1 2NY

1 Human intestinal epithelial Caco-2 cells have been used to investigate the transepithelial permeation of the cardiac glycoside, digoxin.

2 Transepithelial basal to apical [^{3}H]-digoxin flux exceeds apical to basal flux, a net secretion of [^{3}H]-digoxin being observed. At 200 μM digoxin, net secretory flux (J_{net}) was 10.8 ± 0.6 nmol cm^{-2} h^{-1}. Maximal secretory flux (J_{max}) of vinblastine was 1.3 ± 0.1 nmol cm^{-2} h^{-1}. Cellular uptake of digoxin was different across apical and basal cell boundaries. It was greatest across the basal surface at 1 μM, whereas at 200 μM, apical uptake exceeded basal uptake.

3 Net secretion of [^{3}H]-digoxin was subject to inhibition by digitoxin and bufalin but was not inhibited by ouabain, convallatoxin, and strophanthidin (all 100 μM). Inhibition was due to both a decrease in J_{b-a} and an increase in J_{a-b}. Uptake of [^{3}H]-digoxin at the apical surface was increased by digitoxin and bufalin. All cardiac glycosides decreased [^{3}H]-digoxin uptake at the basal cell surface (except for 100 μM digitoxin).

4 The competitive P-glycoprotein inhibitors, verapamil (100 μM), nifedipine (50 μM) and vinblastine (50 μM) all abolished net secretion of [^{3}H]-digoxin due to both a decrease in J_{b-a} and an increase in J_{a-b}. Cellular accumulation of [^{3}H]-digoxin was also increased across both the apical and basal cell surfaces. 1-Chloro-2,4,-dinitrobenzene (10 μM), a substrate for glutathione-S-transferase and subsequent ATP-dependent glutathione-S-conjugate secretion, failed to inhibit net secretion of [^{3}H]-digoxin. The increase in absorptive permeability P_{a-b} ($=J_{a-b}/C_a$) and cellular [^{3}H]-digoxin uptake upon P-glycoprotein inhibition, showed that the intestinal epithelium was rendered effectively impermeable by ATP-dependent extrusion at the apical surface.

5 A model for [^{3}H]-digoxin secretion by the intestinal epithelium is likely to involve both diffusional uptake and Na^+-K^+ pump-mediated endocytosis, followed by active extrusion at the apical membrane.

Keywords: Digoxin; cardiac glycoside; P-glycoprotein; intestine; epithelium; Caco-2 cells

Introduction

The intestinal permeation of cardiac glycosides such as digoxin and digitoxin has been thought to be solely determined by the relative polarity of the glycoside molecule; thus absorption inversely parallels polarity with digitoxin > acetyldigoxin-> digoxin > lanatoside C > ouabain (Rietbrock & Woodcock, 1989). Lauterbach (1981) has discussed evidence that shows oral absorption of cardiac glycosides is not simply described by simple diffusive processes. A strict inverse relationship between polarity and absorption rate does not hold for all glycosides. Indeed the absorption coefficient of digoxin decreased as a function of time after continued oral dosage in the rat (Lauterbach, 1981). Such discrepancies may be explained by the observations of intestinal secretion of digoxin by Lauterbach (1981) in isolated preparations of intestinal mucosae maintained *in vitro*. Similar observations of high transport rates from blood to mucosal directions of transport of cardiac glycosides have been made for human isolated intestinal mucosae (Damm & Woermann, 1974).

An important problem encountered in digitalis therapy is that associated with a substance-specific elimination pathway which is subject to physiological variability and changes in disease (Rietbrock & Woodcock, 1981). Thus maintenance of stable plasma concentrations at constant dosage has been problematic. For digoxin and other renal-dependent glycosides it has been assumed that the only pharmacokinetically significant route of elimination is renal. Intestinal secretory capacity will not only affect absorption kinetics, but will also contribute to whole body clearance of cardiac glycosides particularly with renal insufficiency and in the elderly.

In human intestinal epithelial Caco-2 cell monolayers, we have previously shown that vinblastine absorption displays a non-linear dependence upon luminal (apical) vinblastine concentration, vinblastine absorption increasing markedly at high substrate concentrations (Hunter *et al.*, 1993a). Such behaviour is related to the presence of a substantial secretory capacity for vinblastine (Hunter *et al.*, 1993a,b). This secretion is inhibited by verapamil, consistent with the expression of the ATP-dependent P-glycoprotein pump at the apical (brush-border) membrane (Hunter *et al.*, 1993a,b). A monoclonal antibody (MRK16) not only directly localizes the protein to the brush-border membrane of Caco-2 cells but also inhibits transepithelial vinblastine secretion (Hunter *et al.*, 1993b). Upon inhibition of P-glycoprotein by verapamil and dideoxyforskolin, vinblastine absorption is increased (Hunter *et al.*, 1993a).

P-glycoprotein acts as an energy-dependent efflux pump for a range of cytotoxic and hydrophobic compounds (Hunter *et al.*, 1993a; Burton *et al.*, 1993; Karlsson *et al.*, 1993). Since P-glycoprotein is normally expressed at the apical surface of intestinal epithelia (Cordon-Cardo *et al.*, 1990) it is likely that P-glycoprotein may function as a natural mechanism in detoxification and to limit intestinal permeation of natural toxic compounds found as normal constituents of diet. It should be noted that anti-cancer cytotoxic agents such as vinblastine were originally isolated from natural sources (plants, microbes).

[1] Author for correspondence.

The Caco-2 cell system has proved an appropriate model system for intestinal epithelial permeability studies (Hidalgo *et al.*, 1989; Artursson & Karlsson, 1991). This human intestinal epithelial cell line expresses functional transporters typical of the *in vivo* epithelium e.g. the H^+-coupled dipeptide carriers at both apical and basolateral membranes (Thwaites *et al.*, 1993a,b) capable of selective absorption of cephalosporins (Dantzig & Bergin, 1990; Inui *et al.*, 1992) and ACE inhibitors which show high oral bioavailability *in vivo*. The retention of both absorptive and secretory capacities (above) typical of the intact intestine *in situ* confirm the suitability of this human model system to determine the mechanistic basis of unusual absorption kinetics.

The purpose of the present study was twofold; first to investigate the kinetics of transepithelial transport of the cardiac glycoside, digoxin, in order to identify the possible existence of a specific secretory pathway in human intestinal cells and second to examine the contribution of P-glycoprotein (MDR1) in such a secretory pathway.

Methods

Cell culture

Caco-2 cells were obtained from Dr I. Hassan (Ciba-Geigy Pharmaceuticals, Horsham, Sussex) and used between passage number 95–114. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g l^{-1} glucose and supplemented with 1% non-essential amino acids, 2 mM L-glutamine, 10% (v/v) foetal calf serum and gentamycin (60 μg ml^{-1}). Cell monolayers were prepared by seeding at high density ($4.4-5.0 \times 10^5$ cells cm^{-2}) onto tissue culture inserts (Transwell polycarbonate filters (Costar)). Cell monolayers were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cell confluence was estimated by microscopy and determination of transepithelial electrical resistance (R_T) using a WPI Evometer fitted with 'chopstick' electrodes (World Precision Instruments, Stevenage, Hertfordshire), measured at 37°C in Krebs buffer (Hunter *et al.*, 1991). Cell layers were typically used when the transepithelial resistance across the monolayer was between 200–300 Ω.

Transepithelial transport experiments

Uptake and transport experiments with digoxin were performed 14–21 days after seeding and 18–24 h after feeding. Transepithelial flux measurements were performed as described previously (Thwaites *et al.*, 1993a). Briefly, the cell monolayers (24.5 mm in diameter) were washed by sequential transfer through 4 beakers containing 500 ml of modified Krebs buffer (all mmol l^{-1}): NaCl 137, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1.0, NaH_2PO_4 0.3, KH_2PO_4 0.3, glucose 10, HEPES/Tris 10 (pH 7.4, 37°C) and placed in 6-well plates, each well containing 2 ml of modified Krebs buffer. Krebs buffer (2 ml; pH 7.4) was placed in the upper filter cup (apical solution) and the filters were incubated for 10 min at 37°C. The experimental composition of the buffers in the apical and basal chambers were identical except where stated otherwise. Radiolabelled [^{3}H]-digoxin or [^{3}H]-ouabain and [^{14}C]-mannitol (0.1 μCi ml^{-1}) were added to either the apical or basolateral chamber and in each case an equivalent concentration of unlabelled substrate was present in the contralateral chamber. For experiments where the unlabelled digoxin concentration was varied and where other drugs were present, equal concentrations were present in both the apical and basolateral bathing solutions. Fluxes in the absorptive (apical-to-basal, J_{a-b}) and secretory (basal-to-apical, J_{b-a}) directions were determined for 1 h (after a 20 min preincubation to establish a state of linear flux) on adjacent paired cell monolayers and are expressed as nmol cm^{-2} h^{-1} or pmol cm^{-2} h^{-1}.

The passive (paracellular) route across the epithelium was estimated by concurrent mannitol flux determinations. Mannitol flux into the contralateral chamber was typically 2% at the end of the incubation period. Flux values of > 5% led to rejection of the monolayer and associated digoxin flux determination. At the end of the incubation period cell monolayers were washed by sequential transfer through 4 beakers containing 500 ml volumes of Krebs buffer (pH 7.4) at 4°C to remove any loosely-associated radiolabel, and removed from the insert. Cell monolayer-associated radiolabel was determined by scintillation counting. Cellular uptake of digoxin is expressed as μM or as a cell/medium (C/M) ratio. Cell height was determined by confocal microscopy and this value was used in the determination of intracellular volume. Determinations of the transepithelial transport of vinblastine sulphate (10 nM – 100 μM vinblastine sulphate with [^{3}H]-vinblastine sulphate as tracer) were made identically to digoxin.

Cellular uptake of ^{86}Rb across apical and basolateral surfaces

Cell monolayers were washed by sequential transfer through 4 beakers containing 500 ml Krebs solution (pH 7.4) at 37°C and placed in fresh 6-well plates containing 2 ml pre-warmed Krebs solution (pH 7.4) in both apical and basolateral compartments. Uptake was initiated by replacing the apical or basal bathing solution with an experimental solution containing ^{86}Rb (1 μCi ml^{-1}). After a 10 min incubation the apical or basal bathing solution was rapidly aspirated and the cell monolayer was washed by sequential transfer through 4 beakers containing 500 ml ice-cold Krebs solution (pH 7.4). Cell-associated ^{86}Rb activity was determined after extraction in distilled water by scintillation spectrometry using the Cherenkov effect (Aiton *et al.*, 1982).

Estimation of Na^+-K^+ pump density using [^{3}H]-ouabain binding

K^+-sensitive ouabain uptake over a 15 min incubation was determined at saturating ouabain concentrations (for pump inhibition) as a measure of Na^+-K^+ pump density (Lamb *et al.*, 1981). The difference in binding (i.e. K^+-sensitive uptake) between a high affinity pump conformation (K^+-free media) and a low affinity pump conformation (15 mM K^+-media) at 15 min gives an estimate of the specific binding to the Na^+/K^+ ATPase (Lamb *et al.*, 1981). Cell monolayers were washed by sequential transfer through 4 beakers containing 500 ml K^+-free Krebs solution (K^+ salts omitted) (pH 7.4) at 37°C and placed in fresh 6-well plates containing 2 ml pre-warmed Krebs solution (pH 7.4) with 0.1 μCi ml^{-1} [^{3}H]-ouabain in either K^+-free Krebs or 15 mM KCl-containing Krebs solution (the basolateral compartment). Alternatively radioactive soak solutions were applied to the apical compartment with K^+-free Krebs solution in the basal compartment. After incubation for 15 min, monolayers were washed 4 times by sequential transfer through ice-cold K^+-free Krebs solution. Cell-associated radioactivity was determined as described for flux determinations.

Materials

[^{14}C]-mannitol (specific activity 50 Ci $mmol^{-1}$) and [^{3}H]-digoxin (specific activity 20 Ci $mmol^{-1}$) were from New England Nuclear (Stevenage, Hertfordshire). [^{3}H]-ouabain (specific activity 50 Ci $mmol^{-1}$), [^{3}H]-vinblastine sulphate (specific activity 16 Ci $mmol^{-1}$) and ^{86}Rb (0.5–10 mCi mg^{-1} rubidium) were from Amersham (Little Chalfont, Buckinghamshire). Cardiac glycosides were from Sigma (Poole, Dorset). All cardiac glycosides were added to Krebs buffer from ethanolic stock solutions except ouabain which was added directly. Cell culture media, supplements and plastic were supplied by Life Technologies (Paisley, Scotland). All other chemicals were from Merck (Poole, Dorset).

Statistics

Results are expressed as mean ± s.e.mean (n). Statistical analysis was performed using Student's unpaired t test or one-way analysis of variance (ANOVA) with a Dunnetts post test for multiple comparisons (Graph-Pad, Instat). Kinetic constants for Michaelis-Menten kinetics were calculated by non-linear regression with the method of least squares (FigP, Biosoft, Cambridge, U.K.).

Results

Transepithelial secretion and cellular accumulation of [^{3}H]-digoxin

Figure 1a shows that in epithelial layers of human intestinal cells basal to apical flux (J_{b-a}) exceeds apical to basal flux (J_{a-b}) of [^{3}H]-digoxin; that is Caco-2 epithelia are capable of net secretion of digoxin from basal to apical surfaces. The absorptive permeability of digoxin ($P_{a-b} = J_{a-b}/C_a$; where C_a is apical solution concentration) at 1 μM digoxin was 0.87 ± 0.11 cm h$^{-1} \times 10^{-2}$ ($n = 12$). Concurrent measurement of the absorptive permeability P_{a-b} for [^{14}C]-mannitol gave 0.18 ± 0.01 cm h$^{-1} \times 10^{-2}$ ($n = 12$). There is no asymmetry in the measured mannitol fluxes, P_{b-a} not being statistically significant from P_{a-b} (0.17 ± 0.01 cm h$^{-1} \times 10^{-2}$ ($n = 12$), $P > 0.05$, NS.). Net secretory flux of digoxin ($J_{net} = J_{b-a} - J_{a-b}$) was concentration-dependent, but failed to reach saturation even at 200 μM digoxin, which was the highest concentration used due to solubility in the modified Krebs solution. Though absorptive permeability at 200 μM digoxin was very similar to that observed at 1 μM (1.15 ± 0.17 cm h$^{-1} \times 10^{-2}$ ($n = 11$)), that for secretory permeability ($P_{b-a} = J_{b-a}/C_b$; where C_b is basal solution concentration) was reduced as digoxin concentration was raised (7.38 ± 0.52 cm h$^{-1} \times 10^{-2}$ ($n = 12$) (at 1 μM) versus 5.41 ± 0.34 cm h$^{-1} \times 10^{-2}$ ($n = 11$), $P < 0.05$) suggesting the existence of a saturable process. It should be noted that even with inclusion of 200 μM digoxin in the incubation media, transepithelial mannitol permeability was not altered ($P_{a-b} = 0.16 \pm 0.01$ cm h$^{-1} \times 10^{-2}$ and $P_{b-a} = 0.16 \pm 0.01$ cm h$^{-1} \times 10^{-2}$ ($n = 11$), $P > 0.05$, NS versus controls at 1 μM digoxin), confirming that the high concentrations of cardiac glycoside do not adversely affect epithelial integrity over the time course of the experiment. Calculation of an apparent K_m and J_{max} for net digoxin secretion (Figure 1a) gave 0.65 ± 0.18 mM and 46.4 ± 10.4 nmol cm^{-2} h^{-1} respectively. This level of net secretion and that observed at the maximal concentration experimentally (200 μM digoxin) appears incompatible with the maximal secretory capacity observed for P-glycoprotein substrates such as vinblastine previously reported in Caco-2 intestinal epithelia (1.3 nmol cm^{-2} h^{-1}, Hunter et al., 1993a,b). If vinblastine and digoxin secretion are mediated by an identical pathway it would be expected that the maximal secretory capacity would be identical. Accordingly, the maximal vinblastine secretion and digoxin secretion were assessed in an identical cell batch. For vinblastine secretion the measured K_m and J_{max} were 13.8 ± 2.8 μM and 1.3 ± 0.1 nmol cm^{-2} h^{-1}. (d.f. = 15). In contrast, the apparent K_m and J_{max} for digoxin in the same cell batch were 0.26 ± 0.11 mM and 23.3 ± 6.1 nmol cm^{-2} h^{-1}.

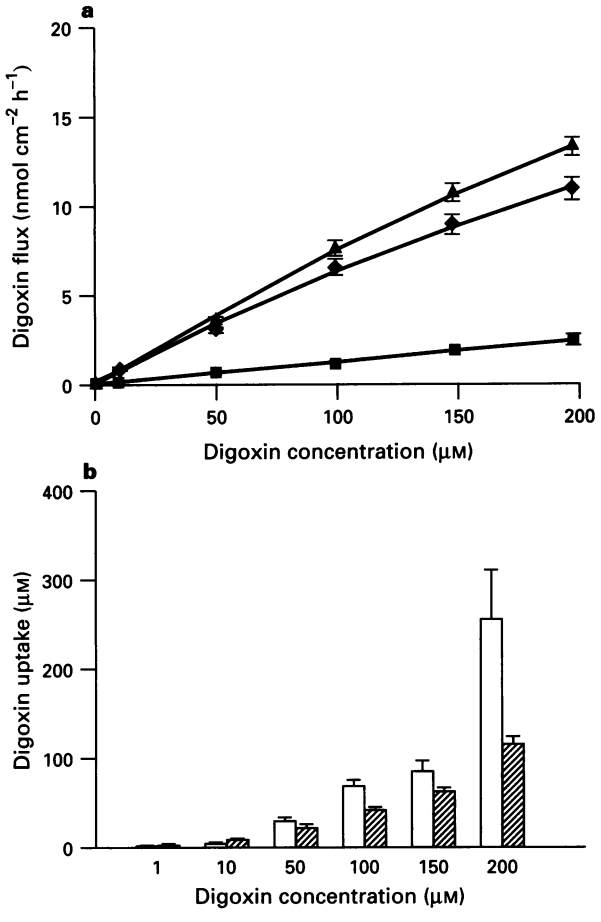

Figure 1 Concentration-dependence of secretion and cellular accumulation of digoxin by human intestinal Caco-2 cell monolayers. (a) Transepithelial [^{3}H]-digoxin fluxes (0.1 μCi/ml^{-1}, 3.6 nM) were determined in adjacent paired monolayers for apical to basal flux (J_{a-b}) (■) and basal to apical flux (J_{b-a}) (▲). Net secretory flux was = $J_{b-a} - J_{a-b}$(◆). Increasing concentrations of unlabelled digoxin were present in both apical and basal bathing fluid compartments. All data mean ± s.e., $n = 11-12$ epithelia per point. (b) Cellular accumulation of digoxin after 1 h incubation with increasing digoxin concentrations at the apical (open columns) and basal (hatched columns) membrane surfaces. [^{3}H]-digoxin present in either apical or basal bathing solution. All data mean ± s.e., $n = 11-12$ epithelia.

Figure 1b shows the concentration-dependence of apparent [^{3}H]-digoxin uptake by Caco-2 cells from apical or basal bathing solutions. At low bathing solution digoxin concentration e.g. 1 μM there was an asymmetry in that [^{3}H]-digoxin uptake at the basal surface exceeded that at the apical surface; expressed as a cell to medium ratio (C/M) and assuming uniform distribution of the label within the cytosol, the C/M ratio for apical [^{3}H]-digoxin was 0.63 ± 0.01, whereas for basal [^{3}H]-digoxin it was 1.57 ± 0.13 ($n = 12$), $P < 0.01$. In comparison, that for [^{14}C]-mannitol was 0.26 ± 0.03 and 0.17 ± 0.01 at the apical and basolateral membranes respectively after the prolonged 1 h incubation for flux determinations. As digoxin concentration is raised to 200 μM these ratios change to 1.26 ± 0.28 and 0.58 ± 0.01 respectively. Binding of digoxin to the filter was $0.034 \pm 0.009\%$ ($n = 3$) and $0.036 \pm 0.006\%$ ($n = 3$) of total radioactivity added in the apical and basal compartments respectively (at 1 μM digoxin). This compares with $0.123 \pm 0.006\%$ ($n = 6$) and $0.402 \pm 0.035\%$ ($n = 6$) of total radioactive digoxin into the cells from the apical and basolateral compartments respectively (at 1 μM digoxin). As filter binding was small in comparison to accumulation within the cells, showed no asymmetry and may not be representative of the level of filter binding which occurs when cells are present, cellular accumulation data were not modified for filter binding.

Effect of various cardiac glycosides on transepithelial [^{3}H]-digoxin secretion and cellular accumulation

Figure 2 and Table 1 demonstrate a partial structure-activity relationship for the ability of various cardiac glycosides to affect transepithelial [^{3}H]-digoxin secretion. Ouabain, strophanthidin and convallatoxin (all at 20 μM and 100 μM) failed to inhibit digoxin secretion. In contrast digitoxin (at 20 μM and 100 μM) and bufalin (100 μM) were effective in inhibition of net [^{3}H]-digoxin secretion. This inhibitory action by digitoxin was

due to both a reduction in J_{b-a} and an increase in absorptive flux (J_{a-b}) (Figure 2a). The absorptive permeability for [^{3}H]-digoxin increased from 0.47 ± 0.06 cm $h^{-1} \times 10^{-2}$ ($n=4$) to 3.51 ± 0.72 cm $h^{-1} \times 10^{-2}$ ($n=3$), $P<0.01$ in the presence of 100 μM digitoxin. This increase occurred in the absence of change in [^{14}C]-mannitol permeability measured concurrently (control P_{a-b} for mannitol $= 0.23 \pm 0.01$ cm $h^{-1} \times 10^{-2}$ ($n=4$), plus 100 μM digitoxin P_{a-b} for mannitol $= 0.24 \pm 0.19$ cm $h^{-1} \times 10^{-2}$ ($n=3$) $P>0.05$, NS), confirming that epithelial integrity was not affected in these conditions. The ability of digitoxigenin, the aglycone of digitoxin, to inhibit [^{3}H]-digoxin secretion was also investigated. At 20 μM and 100 μM digitoxigenin failed to inhibit secretory [^{3}H]-digoxin permeability (P_{net} in controls was 6.80 ± 0.53 cm $h^{-1} \times 10^{-2}$, plus digitoxigenin 5.92 ± 0.15 cm $h^{-1} \times 10^{-2}$ and 6.70 ± 0.27 cm $h^{-1} \times 10^{-2}$ respectively, all $n=4$, NS versus control data).

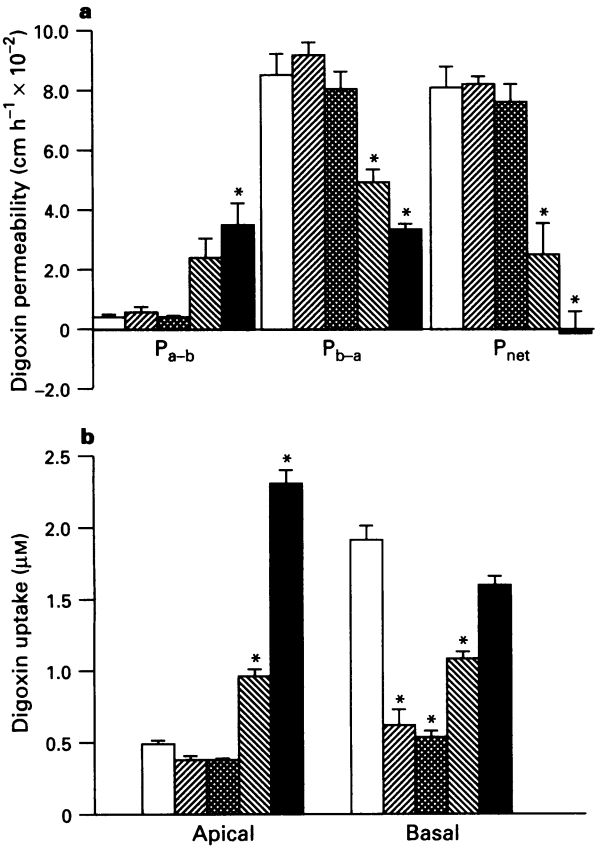

Figure 2 Transepithelial [^{3}H]-digoxin transport and cellular accumulation in the presence of ouabain and digitoxin. (a) Transepithelial and net secretory transport of [^{3}H]-digoxin (3.6 nM) alone (open columns) and in the presence of 20 μM ouabain (upward sloping hatched columns,/), 100 μM ouabain (cross-hatched columns), 20 μM digitoxin (downward sloping hatched columns,\), and 100 μM digitoxin (solid columns). (b) Cellular accumulation of [^{3}H]-digoxin in the presence of ouabain and digitoxin as (a). Data are the mean $\pm$ s.e., $n=3-4$ epithelial monolayers per data point.

Since digoxin itself fails to compete effectively with [^{3}H]-digoxin for secretion, an absence of inhibition as with ouabain (Figure 2a) may reflect a low-affinity interaction with the secretory transport. Accordingly, transepithelial [^{3}H]-ouabain fluxes were measured to confirm that no secretion of this cardiac glycoside was present. At 7 nM ouabain P_{a-b} was just 0.23 ± 0.06 cm $h^{-1} \times 10^{-5}$ ($n=4$), whilst P_{b-a} was 0.15 ± 0.03 cm $h^{-1} \times 10^{-5}$ ($n=4$). These permeabilities were not altered by inclusion of 100 μM ouabain, digoxin or digitoxin. Thus there is no evidence of transepithelial secretion, the absolute permeability of this molecule is markedly lower than either digoxin or mannitol and the transepithelial transport is consistent with an entirely passive mechanism.

Figure 2b and Table 1 show that cardiac glycosides have opposite actions on [^{3}H]-digoxin uptake at the apical and basolateral membrane faces. Digitoxin at 20 μM and 100 μM and bufalin at 100 μM markedly increased uptake across the apical membrane; ouabain, strophanthidin and convallatoxin (at 100 μM) were without effect. In contrast ouabain, strophanthidin and convallatoxin all depressed [^{3}H]-digoxin uptake at the basolateral membrane. At the basolateral membrane face, digitoxin (100 μM) had no effect on uptake and bufalin (100 μM) depressed [^{3}H]-digoxin uptake compared to control levels, but gave an increase in cellular uptake at the apical membrane in a concentration-dependent manner in comparison to those cardiac glycosides that did not inhibit digoxin transepithelial secretion.

It is likely that the ability of cardiac glycosides to reduce [^{3}H]-digoxin uptake at the basolateral membrane face is related to their ability to bind to and inhibit the Na^+-K^+ pump. Accordingly, the polarity of expression of the Na^+-K^+ ATPase was investigated by determining the location of ouabain-sensitive ^{86}Rb(K) uptake (Table 2). ^{86}Rb(K) uptake across the basolateral membrane was 5 fold higher than that across the apical membrane. A ouabain-sensitive component of ^{86}Rb(K) uptake was observed only at the basolateral sur-

Table 1 Effect of cardiac glycosides on the transport and cellular uptake of digoxin (1 μM) ($n=3-11$)

	Relative potency (digitoxigenin = 1)	*P_{net} ($cm\,h^{-1} \times 10^{-2}$)*	*Apical uptake (μM)*	*Basal uptake (μM)*
Control	0.3 to 6.3	7.70 $\pm$ 0.31 (100%)	0.46 $\pm$ 0.01 (100%)	1.59 $\pm$ 0.09 (100%)
Digoxin (100 μM)		7.33 $\pm$ 0.43 (95.2%)	0.63 $\pm$ 0.10 (137%)	0.41 $\pm$ 0.02 (25.8%)*
Strophanthidin (20 μM)	0.1 to 0.5	8.11 $\pm$ 0.65 (105%)	0.36 $\pm$ 0.03 (78.3%)	0.44 $\pm$ 0.04 (27.7%)*
Strophanthidin (100 μM)		6.86 $\pm$ 0.52 (89.1%)	0.38 $\pm$ 0.00 (82.6%)	0.49 $\pm$ 0.02 (30.8%)*
Convallatoxin (20 μM)	1.3 to 3.8	8.77 $\pm$ 0.38 (114%)	0.31 $\pm$ 0.00 (67.4%)	0.48 $\pm$ 0.01 (30.2%)*
Convallatoxin (100 μM)		7.88 $\pm$ 0.69 (102%)	0.35 $\pm$ 0.02 (76.1%)	0.45 $\pm$ 0.03 (28.3%)*
Bufalin (20 μM))	10.7	7.22 $\pm$ 0.47 (93.8%)	0.53 $\pm$ 0.04 (115%)	0.65 $\pm$ 0.03 (40.9%)*
Bufalin (100 μM)		2.19 $\pm$ 0.07 (28.4%)*	0.90 $\pm$ 0.03 (196%)*	1.01 $\pm$ 0.04 (63.5%)*

Relative potencies of cardiac glycosides for inotropic activity from Thomas *et al.* (1990). * Significantly different from control values: $P<0.05$.

face. Secondly, the polarity of K^+-sensitive [^{3}H]-ouabain uptake at the apical and basal cell surfaces was examined (Table 3). Substantial K^+-sensitive binding of ouabain was restricted to the basal cell surfaces. At 1 μM the calculated Na^+-K^+ pump site density was $4.62 \pm 0.03 \times 10^6$ sites/cell.

Effects of inhibitors on transepithelial [^{3}H]-digoxin secretion and on cellular uptake

In order to test whether the secretion of [^{3}H]-digoxin by Caco-2 intestinal epithelia was mediated by P-glycoprotein, the effects of pharmacological modulators of P-glycoprotein (Ford & Hait, 1990; Gottesman & Pastan, 1993) on [^{3}H]-digoxin fluxes were determined (Figures 3 and 4). Verapamil (100 μM), nifedipine and vinblastine (both 50 μM) all eliminated [^{3}H]-digoxin secretion by an inhibition of $J_{b\text{-}a}$ and an increase in $J_{a\text{-}b}$. The absorptive permeability for [^{3}H]-digoxin increased from 0.59 ± 0.05 cm $h^{-1} \times 10^{-2}$ ($n=12$) to 4.19 ± 0.50 cm $h^{-1} \times 10^{-2}$ ($n=12$), $P<0.001$ in the presence of 100 μM verapamil, to 3.92 ± 0.22 cm $h^{-1} \times 10^{-2}$ ($n=11$), $P<0.001$ in the presence of 50 μM nifedipine and to 6.61 ± 0.79 cm $h^{-1} \times 10^{-2}$ ($n=11$), $P<0.001$ in the presence of 50 μM vinblastine. This increase occurred in the absence of equivalent change in [^{14}C]-mannitol permeability measured concurrently (not shown). Cellular uptake of [^{3}H]-digoxin at both apical and basolateral membrane faces was increased after competitive inhibition of P-glycoprotein. The increase in basal uptake of [^{3}H]-digoxin uptake in these conditions, contrasts to that seen with cardiac glycosides including digitoxin (above). These features (increase in cellular uptake, inhibition of secretion) of competitive inhibition of P-glycoprotein are observed for [^{3}H]-vinblastine transport mediated by P-glycoprotein in Caco-2 intestinal epithelia.

Figure 4 shows the concentration-dependent inhibition of [^{3}H]-digoxin transport and cellular uptake by verapamil. Half-maximal inhibition of J_{net}, and half-maximal stimulation of intracellular [^{3}H]-digoxin uptake were seen at equivalent verapamil concentrations of 10–20 μM verapamil. The K_i for inhibition of vinblastine secretion by verapamil in Caco-2 epithelia has previously been determined to be 17 μM (Hunter *et al.*, 1993b).

In order to test whether secretion of glutathione conjugates are involved in digoxin secretion the inhibitory actions of a saturating concentration of 1-chloro 2,4,dinitrobenzene (CDNB) (Elferink *et al.*, 1993) were examined. CDNB is a substrate for glutathione-S-transferase, glutathione-S-dinitrophenylglutathione (GS-DNP) then being subject to ATP-dependent secretion in Caco-2 epithelial cells (Elferink *et al.*, 1993). Basal to apical transport of [^{3}H]-digoxin (1 μM) was unaltered by 10 μM CDNB, whilst absorptive permeability was slightly increased (Table 4). Net secretion was thus unaltered. CDNB (10 μM) had no effect on the basal accumulation whilst apical uptake was slightly increased.

Table 2 Ouabain-sensitive ^{86}Rb (K) uptake across apical and basolateral membrane faces

	Apical uptake (mmol l^{-1} cell water 10 min^{-1})	*Basal uptake* (mmol l^{-1} cell water 10 min^{-1})
Total uptake	7.20 ± 0.28	35.48 ± 0.77
Ouabain-sensitive uptake	0.25 ± 0.35	15.00 ± 0.86

^{86}Rb uptake was determined over 10 min incubations as described in Methods ($n=4$).

Table 3 K^+-sensitive binding of [^{3}H]-ouabain at the apical and basal faces of Caco-2 epithelial cell monolayers

	Apical uptake (pmol cm^{-2})	*Basal uptake* (pmol cm^{-2})
Ouabain (0.25 μM)	0.016 ± 0.003	2.63 ± 0.21
Ouabain (1 μM)	0.046 ± 0.018	3.26 ± 0.02

[^{3}H]-ouabain binding was determined over 15 min incubations as described in Methods. Binding is measured at 0.25 and 1 μM unlabelled ouabain ($n=4$).

Discussion

Several features displayed by transepithelial secretion of digoxin in human intestinal Caco-2 monolayers are entirely compatible with data from both *in vitro* excised gut preparations and by *in vivo* perfusion studies (see Lauterbach, 1981); thus net secretion of the neutral molecule digoxin is active, highly specific and a saturable process. In addition, cellular uptake of [^{3}H]-digoxin is most pronounced from the basal (blood) side as opposed to the apical (lumen) side of the epithelium.

Digoxin secretion is largely unaffected by the inclusion of saturating concentrations of CDNB, which is subjected to glutathione-S-conjugation and secretion (Elferink *et al.*, 1993). Digoxin is thus unlikely to be a substrate, though ATP-hy-

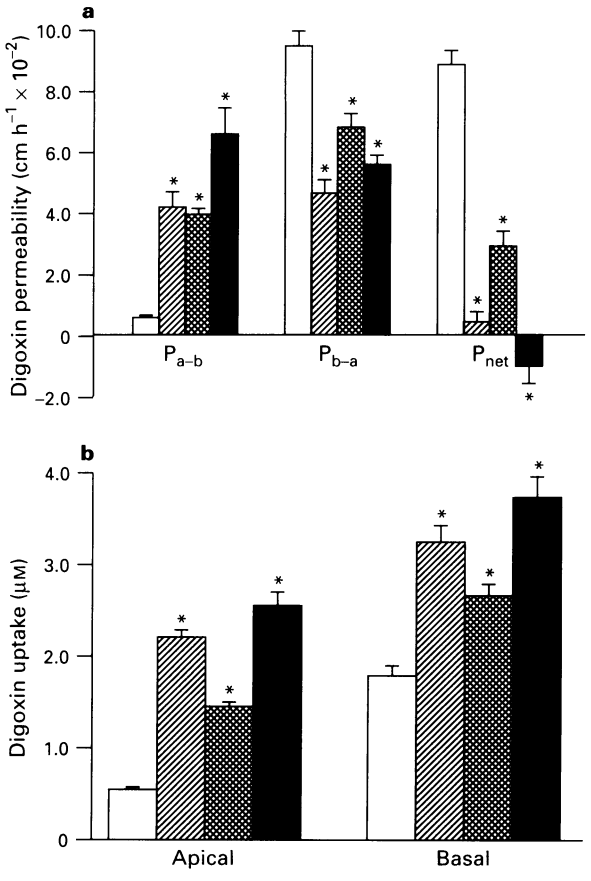

Figure 3 Transepithelial [^{3}H]-digoxin transport and cellular accumulation in the presence of inhibitors of P-glycoprotein. (a) Transepithelial and net secretory transport of [^{3}H]-digoxin (3.6 nM) alone (open columns) and in the presence of 100 μM verapamil (hatched columns), 50 μM nifedipine (cross-hatched columns), and 50 μM vinblastine (solid columns). (b) Cellular accumulation of [^{3}H]-digoxin in the presence of verapamil, nifedipine and vinblastine, columns as in (a). Data are the mean ± s.e., $n=3-4$ epithelial monolayers.

drolysis by a dinitrophenyl-S-glutathione ATPase is stimulated by doxorubicin, daunomycin and vinblastine suggesting that these weakly cationic amphiphiles are substrates (Awasthi *et al.*, 1993).

There is convincing evidence for expression of P-glycoprotein (MDR1) in Caco-2 epithelial cell monolayers. Monoclonal antibodies identify an appropriate protein by Western-blotting (Peters & Roelofs, 1992) whilst immunocytochemical studies (using MRK16) stain the brush-border of Caco-2 cells (Hunter *et al.*, 1993b). Measurements of transepithelial transport of typical P-glycoprotein substrates such as vinblastine show a high-capacity secretion (Hunter *et al.*, 1993a,b), which is inhibited by competitive substrates such as verapamil, nifedipine and taxotere, and partially by MRK16. The present data show that the cardiac glycoside, digoxin, is also subject to transepithelial secretion that is inhibited by P-glycoprotein substrates. Furthermore inhibition of digoxin secretion occurs at a similar K_i to that observed for inhibition of vinblastine secretion. Thus it is likely that at least part of transepithelial digoxin secretion by human Caco-2 intestinal cells is mediated *via* P-glycoprotein.

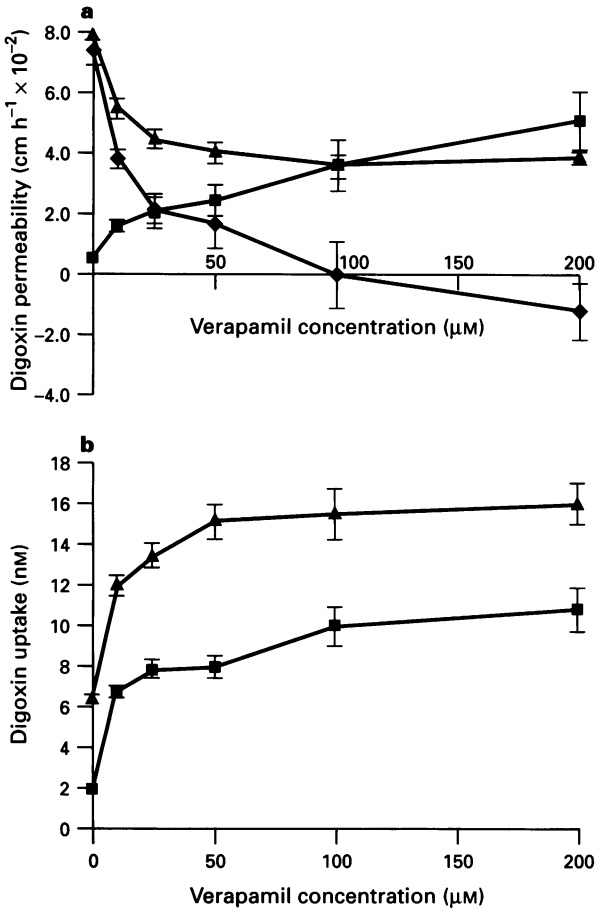

Figure 4 Concentration-dependence of (a) inhibition of [^{3}H]-digoxin transepithelial transport by verapamil. [^{3}H]-digoxin (3.6 nM) was used to measure apical to basal flux (J_{a-b}) (■) and basal to apical flux (J_{b-a}) (▲). Net secretory transport (◆). Increasing concentrations of verapamil were present in both apical and basal bathing solutions. (b) Concentration-dependence of verapamil on cellular [^{3}H]-digoxin; apical accumulation (■), basal accumulation (▲). All data mean ± s.e., $n = 6-7$ epithelial monolayers per point.

There is, however, a discrepancy between the maximal transport rate of digoxin compared to that of vinblastine. Do separate transporters with similar characteristics exist, or does cytosolic vinblastine exert a self-inhibitory action (*via* disruption of microtubules)? It should be noted that MRK16 inhibition of vinblastine secretion is incomplete, thus antibody inhibition studies would not be definitive in determining the existence of multiple secretory pathways (Hunter *et al.*, 1993b). In transgenic mice in which the mouse mdr1a gene has been disrupted, the tissue distribution of digoxin is increased in tissues such as brain, kidney and liver where P-glycoprotein is expressed (Schinkel *et al.*, 1995). In mouse tissue, two genes mdr1a and mdr1b subserve ATP-dependent drug extrusion. Whether mdr1b preferentially transports substrates such as digoxin is not known, but this might also indicate multiple mechanisms.

Digoxin has been shown to be a substrate for renal P-glycoprotein (deLannoy & Silverman, 1992b). In normal and transfected renal LLCPK$_1$ cells, a transepithelial secretion of digoxin was evident that was inhibited by P-glycoprotein substrates including cyclosporin (Tanigawara *et al.*, 1992; Ito *et al.*, 1993; Okamura *et al.*, 1993). *In vivo* urinary deposition of digoxin may also be inhibited by P-glycoprotein substrates (deLannoy *et al.*, 1992a; Speeg *et al.*, 1992). On balance, it seems likely that similar cellular mechanisms (ATP-dependent extrusion) operate in both intestinal and renal epithelia.

P-glycoprotein function alters the absorption kinetics of a substrate such as vinblastine (Hunter *et al.*, 1993a). Apical (lumen) to basal (blood) permeability of vinblastine increased as vinblastine concentration was increased and saturation of the transporter occurred. Competitive substrates including verapamil, dideoxyforskolin, nifedipine and taxotere also increased absorptive vinblastine permeability. Similar increases are also reported here for digoxin absorptive permeability. It should be noted that since digoxin has relatively low affinity for P-glycoprotein compared to vinblastine or verapamil, full saturation of the efflux mechanism by digoxin was not achieved. The absorptive permeability of digoxin (measured at 10 μM) gave 0.59×10^{-2} cm h^{-1}; this value is comparable to permeability values for relatively hydrophilic compounds such as mannitol (present data), acetylsalicylic acid (0.86×10^{-2} cm h^{-1}) and practolol (0.32×10^{-2} cm h^{-1}) in the identical Caco-2 cell system (Artursson & Karlsson, 1991). Thus the absorption coefficient of the relatively lipophilic digoxin appears anomalous. The present data show clearly that inhibition of P-glycoprotein mediated transport by competitive inhibitors such as verapamil and nifedipine will result in a marked increase in the absorptive permeability of digoxin. Thus the operation of an active extrusion mechanism (such as P-glycoprotein) renders the intestinal epithelium relatively impermeable to digoxin.

Whether P-glycoprotein ATP-dependent export will affect absorption kinetics will be dependent not only upon the passive permeability of the apical membrane to the substrate, but also its affinity for the active transport site and the maximal capacity of P-glycoprotein contained in the apical membrane.

Table 4 Effect of 10 μM 1-chloro 2,4-dinitrobenzene (CDNB) on the transport and cellular uptake (cellular uptake expressed as a cell/medium ratio (C/M)) of [^{3}H]-digoxin (1 μM) ($n = 12$)

	P_{a-b} (cm h^{-1} x 10^{-2})	P_{b-a} (cm h^{-1} x 10^{-2})	P_{net} (cm h^{-1} x 10^{-2})	*Basal uptake*	*Apical uptake*
Control	0.46 ± 0.04	9.01 ± 0.44	8.55 ± 0.44	0.90 ± 0.07	0.38 ± 0.04
CDNB 10 μM	0.69 ± 0.09*	8.48 ± 0.53	7.75 ± 0.55	1.02 ± 0.05	0.69 ± 0.09*

* Significantly different from control values: $P < 0.05$.

A simple pump-leak balance will exist at the apical membrane. Whereas digitoxin is an effective inhibitor of digoxin secretion it is not subjected to renal clearance (Rietbrock & Woodcock, 1989) and has a near complete absorption profile. In this case the lipophilicity of the molecule will ensure high diffusional fluxes at the luminal concentrations in normal dosage form.

There is a well described pharmacokinetic interaction between digoxin and quinidine, cytostatics, verapamil or nifedipine which is thought to be based on renal clearance (Rietbrock & Woodcock, 1989). Plasma digoxin is raised in these situations and since this is based on P-glycoprotein inhibition, an increased intestinal absorption is likely to contribute to this phenomenon in addition to decreased renal clearance.

Secretion of digoxin across the epithelium requires that the cardiac glycoside enters the cytosol across the basolateral membrane, before being subject to ATP-dependent secretion at the apical membrane. Cellular uptake of cardiac glycosides at the basolateral membrane must in part result from interaction with the Na^+-K^+ pump. Lamb & Ogden (1982) have convincingly shown that cellular accumulation of cardiac glycosides such as ouabain and digoxin in Hela cells results from internalization of the Na^+-K^+ pump/digitalis complex from the plasma membrane to endomembrane compartments. In acidic conditions it is envisaged that the glycoside dissociates from the pump resulting in detoxification of the Na^+-K^+ pump which then recycles to the plasma membrane. Cardiac glycoside may then be lost from the cell by diffusion or exocytosis. In epithelial Caco-2 cells the Na^+-K^+ pump is localized to the basolateral membrane domain. Our present [^{3}H]-ouabain uptake data (ouabain is not subject to secretion), show that cellular binding and uptake is predominantly restricted to the basolateral surface. This is confirmed by the measurement of ouabain-sensitive K^+ uptake which is restricted to the basal surface of Caco-2 monolayers. Other cardiac glycosides are capable of competitively inhibiting ouabain uptake suggesting that a similar mechanism must exist for all cardiac glycosides. For [^{3}H]-digoxin, ouabain reduces basolateral uptake, confirming that uptake at this cellular border involves interaction with the Na^+-K^+ pump. Analysis of the dose-dependent uptake of digoxin suggests both a saturable uptake (*via* the Na^+-K^+ pump) and a non-saturable process (diffusion). Concurrent inhibition of P-glycoprotein (with verapamil and nifedipine) increases the level of cytosolic uptake of [^{3}H]-digoxin at the basolateral membrane face suggesting that continual recycling of the Na^+-K^+ pump continues to deliver [^{3}H]-digoxin to acidic endosomes and hence, by diffusion, to the cytosol. Detoxification of epithelial cells expressing P-glycoprotein involves a two-step process, first cellular uptake (mediated by the Na^+-K^+ pump at the basolateral membrane), followed by ATP-dependent extrusion across the apical membrane.

The present data show that there is a clear difference in the molecular specificity of cardiac glycosides for P-glycoprotein-mediated transepithelial secretion and for their inotropic activity (and thus inhibition of Na^+-K^+ pump activity). Whereas ouabain, strophanthidin, and convallatoxin were ineffective in inhibition of [^{3}H]-digoxin secretion, all were effective in reducing cellular uptake of [^{3}H]-digoxin across the basolateral membrane. Though no secretion of ouabain was noted we cannot exclude a low affinity interaction of strophanthidin and convallatoxin with the secretory pump. It is apparent that the major difference in structure allowing interaction with P-glycoprotein will relate to the genin structure, though since digitoxigenin does not inhibit the net secretion of digoxin, the sugar moieties are also important. The presence of a mechanism that reduces cytosolic accumulation of cardiac glycosides such as digoxin has implications for the action of cardiac glycosides and their relative toxicity at the cellular level. Co-expression of P-glycoprotein function at a cellular site with the molecular site of cardiac glycoside action may give rise to resistance to cardiac glycosides. Tissue levels of digoxin are increased in mdr1a deficient mice in a number of tissues including the heart (Schinkel *et al.*, 1995). Since therapeutic regimes involve chronic exposure to cardiac glycosides (and other substrates) more work is needed to define such interactions and to investigate their chronic response to sustained levels of cardiac glycoside.

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