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# Evidence for a non-MDR1 component in digoxin secretion by human intestinal Caco-2 epithelial layers

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**Abstract**

Caco-2 epithelial layers were used as a model to re-evaluate the mechanism(s) by which intestinal digoxin absorption is limited by its active secretion back into the lumen. It is widely recognised that intestinal secretion of digoxin is mediated by the ATP-binding cassette (ABC) transporter Multidrug Resistance 1, MDR1. In *MDR1*-transfected Madin-Darby canine kidney, MDCKII , cell monolayers, digoxin secretion was reduced by the MDR1 inhibitor cyclosporin A, whereas no inhibition was seen in the presence of MK-571, 3-([(3-(2-[7-chloro-2-quinolinyl]ethyl)phenyl]-[(3-dimethylamino-3-oxoprphyl)-thio)-methyl]-thio) propanoic acid, a Multidrug Related Protein (MRP) inhibitor. In contrast, digoxin secretion by Caco-2 epithelia was significantly inhibited by both cyclosporin A and MK-571, suggesting that an additional non-MDR1 component may contribute to this transport. Since digoxin secretion by *MRP2*-transfected MDCKII monolayers was increased by only 1.2-fold relative to controls, it is likely that the contribution of MRP2 to digoxin secretion by Caco-2 cells is negligible. An additional MK-571-sensitive secretory pathway for digoxin, together with MDR1, is likely to mediate digoxin secretion in Caco-2 epithelia.
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*Keywords:* ABC transporter; Caco-2 cell; Digoxin; Intestinal transport; MDR1; P-glycoprotein; MRP2

## 1. Introduction

ABC (ATP binding cassette) proteins comprise a superfamily of ATP-dependent membrane transporters that includes the MDR (Multidrug Resistance) and MRP (Multidrug-Related Protein) subfamilies. MDR1 (ABCB1 or P-glycoprotein) is expressed in normal tissues and is localised to the apical membranes of epithelial cells at a number of sites, including the small intestine, colon, liver, and kidney tubules (Cordon-Cardo et al., 1990). This protein acts as an efflux pump sub-serving two important functions; first, as a natural mechanism in detoxification by ATP-dependent active secretion of substrates at excretory surfaces, and second by limiting the absorption of natural toxic compounds found as normal constituents of diet across the intestinal wall (Hunter et al., 1993a,b). Humans express only one MDR1 protein, which is thought to perform the same function as the two Mdr1 subtypes (Mdr1a and Mdr1b) found in rodents. Studies using knockout mice deficient in both *Mdr1a* and *Mdr1b* genes show that in the absence of mdr1 excretory function, intravenous injections of Mdr1 substrates such as digoxin, vinblastine, and cyclosporin A (Schinkel et al., 1994, 1995, 1996, 1997; Mayer et al., 1996), show altered pharmacokinetics with reduced whole body clearance, raised plasma concentrations, and increased tissue accumulation in those locations where P-glycoproteins normally limit drug uptake, in particular the brain (Schinkel et al., 1995, 1996, 1997).

Using human intestinal epithelial Caco-2 cell monolayers, with vinblastine as a model substrate, we demonstrated previously that vinblastine is subject to net secretion in the blood-to-lumen (basal-to-apical) direction (Hunter et al., 1993a). Furthermore, absorption displayed a non-linear dependence upon luminal (apical) vinblastine concentration, with the absorptive flux increasing markedly at high substrate concentrations (Hunter et al., 1993a). Such behaviour is the direct consequence of a finite secretory capacity for vinblastine mediated via multidrug resistance proteins such as MDR1 (Hunter et al., 1993a,b). Inhibition of secretion by an MDR1 inhibitor, verapamil, increases the absorptive permeability of vinblastine (Hunter et al., 1993a,b). Secondary immunofluorescence using an anti-MDR1 monoclonal

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antibody (MRK16) localises the MDR1 protein to the brush-border membrane of Caco-2 cells, and this inhibitory antibody also reduces transepithelial vinblastine secretion when applied to the apical surface (Hunter et al., 1993b).

Absorption of cardiac glycosides has attracted considerable interest for many years, not least because of the narrow therapeutic index of these drugs (Lauterbach, 1981). Importantly, it is now realised that cardiac glycosides such as digoxin are MDR1 substrates; our own work in Caco-2 epithelia has shown verapamil-sensitive net digoxin secretion from basal to apical surfaces, consistent with MDR1 activity (Cavet et al., 1996). In *mdr1a* knockout (−/−) mice there is enhanced accumulation of [$^3$H]digoxin in brain, kidney, liver, testis, and spleen compared with wild type *Mdr1a* (+/+) animals; however in colon and in small intestine no such enhanced cellular accumulation was evident (Schinkel et al., 1995). In *Mdr1b* (−/−) animals, enhancement of tissue accumulation of [$^3$H]digoxin was not seen in any tissue, including brain, suggesting that mdr1a can compensate for absence of Mdr1b in tissues where both are expressed (Schinkel et al., 1997). Using single or double knockout *Mdr1a/b* (−/−) mice, work by Mayer et al. (1996) and Schinkel et al. (1997) has characterised the contribution of both Mdr1a and Mdr1a/b to biliary and intestinal clearance of [$^3$H]digoxin; whereas biliary excretion is only partially reduced in the *Mdr1a/b* (−/−) animals (from 21% to 13.6% of the i.v. dose), intestinal excretion in bile duct cannulated animals was reduced from around 16% to 2% of the total i.v. dose in both *Mdr1a* (−/−) and *Mdr1a/b* (−/−) animals. These data suggest that significant plasticity in MDR-type proteins may exist in different epithelia, and where the effective function is maintained not by one, but several proteins acting cooperatively, perhaps with overlapping specificities.

The purpose of the present study was to investigate the possibility that multiple transport pathways are involved in digoxin secretion by human intestinal epithelial Caco-2 cell monolayers, by using a combination of ABC transporter inhibitors and Madin-Darby canine kidney, MDCKII, cells stably transfected with either *MDR1* or *MRP2*.

## 2. Materials and methods

### 2.1. 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, UK). [$^3$H]vinblastine sulphate (specific activity 16 Ci mmol$^{-1}$) was from Amersham (Little Chalfont, Buckinghamshire, UK). Cell culture media and supplements were from Sigma (Poole, Dorset, UK), and tissue culture plastic flasks and culture inserts were supplied by Costar (High Wycombe, UK). MK571, 3-([(3-(2-[7-chloro-2-quinolinyl]ethyl)phenyl]-[(3-dimethylamino-3-oxoprphyl)-thio)-methyl]-thio) propanoic acid, was supplied by Affiniti Research Products, Exeter, UK) and was made as 9.1 mM stock in DMSO. All other chemicals were supplied by Sigma. Digoxin was added to Krebs' buffer from ethanolic stock solutions.

### 2.2. Cell culture

Caco-2 cells were obtained from Dr. Ian Hassan (Ciba-Geigy Pharmaceuticals, Horsham, Sussex, UK) and were used between passage numbers 95–114. MDCKII-*MDR1* cells are derived from wild-type MDCKII cells and are stably transfected with human *MDR1*, resulting in overexpression of the apical efflux pump P-glycoprotein. MDCKII-*MRP2* cells are similarly derived but are stably transfected with *MRP2*, resulting in expression of human MRP2 at the apical membrane (Evers et al., 1998). Use of both MDCKII-transfected cell lines was kindly authorised by Prof Piet Borst (Netherlands Cancer Institute, Amsterdam) and Dr. Raymond Evers (Institute for Biomedical Research, Frankfurt, Germany). Wild-type cells express basal levels of endogenous canine mdr1, but do not express mrp2 to an appreciable quantity (Evers et al., 1998). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) Glutamax (Gibco), supplemented with foetal calf serum (10% v/v) and glutamine (1% v/v). Wild-type MDCKII cells (Barker and Simmons, 1981) were used as parental controls for MDCKII-*MDR1* or *MRP2* cell layers for transepithelial transport experiments. Transepithelial potential differences were used as an estimate of MDCKII cell monolayer confluence (Barker and Simmons, 1981), and these were measured in Krebs' buffer using a WPI EVOM voltohmeter fitted with 'chopstick' electrodes (World Precision Instruments, Stevenage, Hertfordshire, UK), following iso-osmotic replacement of the basolateral NaCl buffer with choline chloride (basolateral solution electropositive). Monolayers were discarded if the Na:choline diffusion potential difference was $<17.5$ mV.

Caco-2 cells were cultured in DMEM containing glucose (4.5 g l$^{-1}$) and supplemented with non-essential amino acids (1%), L-glutamine (2 mM), foetal calf serum (10%) and gentamicin (30 μg ml$^{-1}$). Cell monolayers were prepared by seeding at high density ($5.0 \times 10^5$ cells cm$^{-2}$) onto tissue culture inserts (Transwell 3401, 12 mm diameter, 0.4 μm pore size uncoated polycarbonate filters, Costar). Cell monolayers were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ in air. Confluence was estimated by microscopy and determination of $R_T$ using a WPI EVOM voltohmeter, measured at 37 °C in Krebs' buffer (Hunter et al., 1993a,b; Cavet et al., 1996, 1997). Monolayers were discarded if $R_T$ was $<200$ Ω cm$^2$.

### 2.3. Transepithelial transport experiments

Uptake and transport experiments with digoxin and vinblastine were performed 14–21 days (Caco-2) or 4 days (MDCKII and transfected derivatives) after seeding, and

18–24 h after replacing the medium. Transepithelial flux measurements were performed essentially as described previously (Cavet et al., 1996, 1997). Briefly, the cell monolayers were washed (four times) in modified Krebs' buffer (all mM): 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 fresh 12-well plates containing 0.8 ml Krebs' buffer in the basal compartment. Krebs' buffer (0.8 ml) was then added to the apical chamber, and the monolayers were allowed to equilibrate for 10 min at 37 °C before determining the pre-experimental $R_T$. The experimental composition of the buffers in the apical and basal chambers was identical. Radiolabelled [$^3$H]digoxin, [$^3$H]vinblastine (both 0.3 μCi ml$^{-1}$), 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 or vinblastine concentrations were varied, or where potential inhibitors were present, equal concentrations of substrate or inhibitor were included in both the apical and basolateral bathing solutions.

For all substrates, fluxes in the absorptive (apical-to-basal, $J_{a-b}$) and secretory (basal-to-apical, $J_{b-a}$) directions were determined for 1 h on adjacent paired cell monolayers and are expressed as nmol cm$^{-2}$ h$^{-1}$ or pmol cm$^{-2}$ h$^{-1}$. Net secretion ($J_{net}$) was calculated from paired monolayers by subtracting the apical-to-basal flux ($J_{a-b}$) from the paired basal-to-apical ($J_{b-a}$) flux. 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 >2% led to rejection of the monolayer and associated flux determinations. At the end of the incubation period, monolayer integrity was verified using $R_T$ determination, then the cell monolayers were washed by sequential transfer through four beakers containing 500 ml volumes of Krebs' buffer (pH 7.4) at 4 °C to remove any loosely associated radiolabel. The monolayers were then removed from their filter cups using a scalpel blade, and placed into a scintillation vial. Cell monolayer-associated radiolabel was determined by scintillation counting. Cellular accumulation of digoxin is expressed as μM, cell height being determined by confocal microscopy to calculate volume.

To determine the inhibitory effects of the monoclonal anti-P-glycoprotein antibody MRK16, Caco-2 cell monolayers were pre-incubated (at 37 °C) for 2 h with 25 μg ml$^{-1}$ of either MRK16 or control isotype-specific mouse IGg2a present at the apical surface. [$^3$H]digoxin and [$^3$H]vinblastine fluxes were then measured.

### 2.4. Statistics

Results are expressed as mean ± S.E.M. ($n$). Statistical analysis was performed using Student's unpaired $t$-test or one-way analysis of variance (ANOVA) with a Bonferroni post-test for multiple comparisons (GraphPad Instat, San Diego, CA). Kinetic constants for Michaelis–Menten kinetics were calculated by non-linear regression with the method of least squares (GraphPad Prism, San Diego, CA).

## 3. Results

### 3.1. Determination of digoxin and vinblastine transport kinetics in Caco-2 cells

To verify net basal-to-apical secretion of digoxin and vinblastine, the absorptive (apical-to-basal; $J_{a-b}$) and secretory (basal-to-apical; $J_{b-a}$) fluxes of both compounds were measured in human intestinal Caco-2 cell monolayers (data not shown). The kinetics of digoxin and vinblastine secretion were determined simultaneously in the same batch of monolayers in order to provide a direct comparison. Net secretion ($J_{net}$) of paired monolayers was calculated by subtracting $J_{a-b}$ from $J_{b-a}$. The calculated maximal secretory capacity ($V_{max}$) of digoxin $J_{net}$ was 8.37 ± 0.20 nmol cm$^{-2}$ h$^{-1}$ ($n$ = 3), compared with 1.25 ± 0.10 nmol cm$^{-2}$ h$^{-1}$ ($n$ = 3) for vinblastine $J_{net}$ (a 6.7-fold difference). The calculated $K_m$ values for net secretion of digoxin and vinblastine were 134.90 and 13.81 μM, respectively, indicating that digoxin is transported with around 10-fold lower affinity than vinblastine. These data are similar to our previously published data in Caco-2 cells, in which we reported the maximal secretory capacity of vinblastine secretion to be 1.2 nmol cm$^{-2}$ h$^{-1}$ (Hunter et al., 1993b), and the maximal secretory capacity for digoxin to be 10.8 nmol cm$^{-2}$ h$^{-1}$ (Cavet et al., 1996). Stephens et al. (2001) also reported a maximal secretory capacity for digoxin secretion across Caco-2 monolayers of 13 nmol cm$^{-2}$ h$^{-1}$.

### 3.2. Contribution of non-MDR1 transporters to digoxin secretion

As well as expressing MDR1 at the apical membrane, Caco-2 cells also express apical MRP2 (Walgren et al., 2000). Fig. 1 shows that the MRP selective inhibitor MK-571 inhibits net secretion of digoxin in Caco-2 cells. This was brought about by a reduction in $J_{b-a}$ and an increase in $J_{a-b}$ (Fig. 1A). Furthermore, MK-571 increased the cellular accumulation of digoxin across both the apical and basolateral cell borders (Fig. 1B). These data are consistent with MK-571 inhibiting an apically located efflux pump, such as MRP2.

MK-571 and the MDR1 inhibitor cyclosporin A were next investigated for their differential ability to inhibit digoxin secretion in Caco-2 monolayers. Fig. 2A shows that 50 μM MK-571 markedly reduced net digoxin secretion in Caco-2 epithelia (by 51%; $P$ < 0.05 vs. controls, $n$ = 4). As above, inhibition of secretion was also accompanied by an increased cellular content of digoxin (Fig. 2B), suggesting

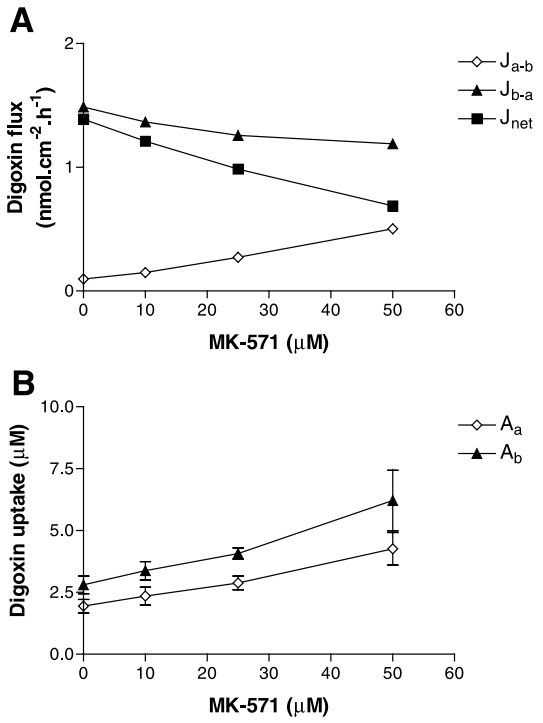


Fig. 1. Partial inhibition of digoxin secretion by MK-571 in Caco-2 epithelia. (A) Transepithelial [$^3$H]digoxin fluxes (10 μM) were measured in the apical-to-basal ($J_{a-b}$) and basal-to-apical ($J_{b-a}$) directions in adjacent paired monolayers. Net secretory flux ($J_{net}$) = $J_{b-a} - J_{a-b}$. (B) Effect of MK-571 upon cellular uptake of digoxin across apical and basal cell borders. Data are mean ± S.E.M. of four epithelial layers or pairs per data point. Error bars lie within data points where not shown.

that MK-571 inhibits digoxin export across the apical membrane of Caco-2 cells. Treatment with 100 μM cyclosporin A had a similar effect to MK-571, causing a marked reduction in digoxin secretion (through both a decrease in $J_{b-a}$ and an increase in $J_{a-b}$), accompanied by an increase in digoxin accumulation across both cell borders. This confirms inhibition of apical MDR1 by cyclosporin A. When used together, MK-571 (50 μM) and cyclosporin A (100 μM) abolished net digoxin secretion altogether (Fig. 2A), the effects of the combined treatment being greater than either inhibitor when used alone. The increase in the cellular accumulation of digoxin caused by the combined treatment was also greater than the effects of either inhibitor when used alone (Fig. 2B). These data indicate that MK-571 and cyclosporin A are inhibiting separate transport systems.

### 3.3. MRK16 inhibition of vinblastine and digoxin transport

The ability of the MDR1 inhibitory antibody MRK16 to inhibit basal-to-apical ($J_{b-a}$; secretory) fluxes of both vinblastine and digoxin (at 10 μM) was tested by preincubating Caco-2 cell layers for two-hours with a control antibody or MRK16 prior to flux measurement (Hunter et al., 1993b). MRK16 caused significant reduction (of 36%) in the $J_{b-a}$ flux of vinblastine (from 1.23 ± 0.20 to 0.79 ± 0.03 nmol $cm^{-2}$ $h^{-1}$; both $n = 12$, $P < 0.05$), which is virtually identical to the reduction reported previously (Hunter et al., 1993b). In contrast, no reduction was seen for digoxin $J_{b-a}$ (0.95 ± 0.03 nmol $cm^{-2}$ $h^{-1}$ in controls, vs. 0.91 ± 0.03 nmol $cm^{-2}$ $h^{-1}$ in the presence of MRK16; both $n = 12$, $P > 0.05$). The differential sensitivity to MRK16 suggests a difference in mechanism between the ability of Caco-2 cells to secrete vinblastine and digoxin (see Discussion).

### 3.4. Selective actions of MK-571 and cyclosporin A on MDR1 and MRP2

To demonstrate that cyclosporin A inhibits MDR1, and to confirm that MK-571 does not inhibit MDR1, we next investigated the effects of these inhibitors on digoxin secretion across monolayers of MDCKII cells stably transfected with human MDR1. First, the bi-directional fluxes of digoxin across wild-type MDCKII monolayers were compared with those across MDR1-transfected MDCKII epithelia, using vinblastine as an internal control (both substrates 10 μM). In the MDR1-transfected cells, net secretion of

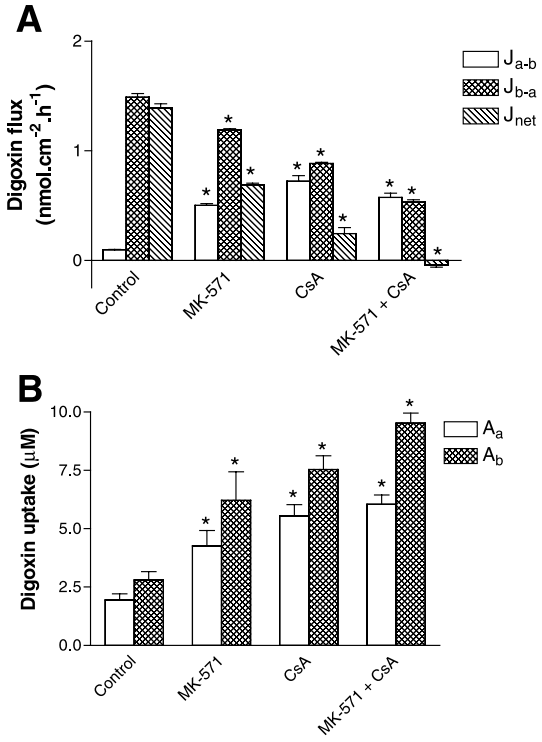


Fig. 2. Additive effects of MK-571 and cyclosporin A upon digoxin secretion in Caco-2 epithelial layers. (A) Transepithelial [$^3$H]digoxin fluxes (10 μM) were measured in the apical-to-basal ($J_{a-b}$) and basal-to-apical ($J_{b-a}$) directions in adjacent paired monolayers. Net secretory flux ($J_{net}$) = $J_{b-a} - J_{a-b}$. MK-571 (50 μM) or cyclosporin A (100 μM) was present in both the apical and basal bathing compartments either alone, or in combination (50 μM MK-571 + 100 μM cyclosporin A). (B) Cellular uptake of digoxin across apical or basal cell borders in the presence and absence of the above inhibitors. Data are the mean ± S.E.M. of four epithelial layers or pairs per column. *$P < 0.05$ significantly different from control layers.

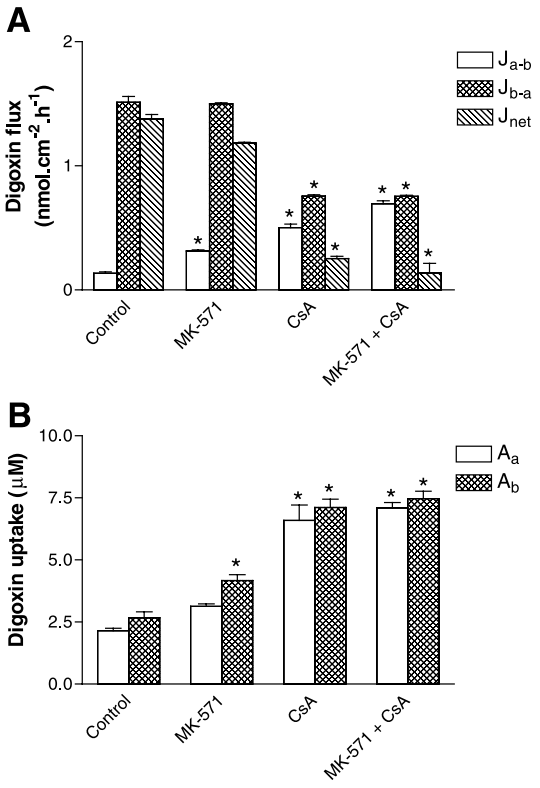


Fig. 3. Sensitivity of digoxin secretion in MDCKII-*MDR1* epithelial layers to the MRP inhibitor MK-571 and the MDR1 inhibitor cyclosporin A. (A) Transepithelial [$^3$H]digoxin fluxes (10 μM) were measured in the apical-to-basal ($J_{a-b}$) and basal-to-apical ($J_{b-a}$) directions in adjacent paired monolayers. Net secretory flux ($J_{net}$)=$J_{b-a}-J_{a-b}$. MK-571 (50 μM) or cyclosporin A (100 μM) was present in both the apical and basal bathing compartments either alone, or in combination (50 μM MK-571 + 100 μM cyclosporin A). (B) Cellular uptake of digoxin across apical or basal cell borders in the presence and absence of the above inhibitors. Data are the mean ± S.E.M. of four epithelial layers or pairs per column. *$P<0.05$ significantly different from control layers.

vinblastine was increased by 3.6-fold (from 0.67 ± 0.07 to 2.39 ± 0.06 nmol cm$^{-2}$ h$^{-1}$; both $n=14$ paired monolayers, $P<0.05$), and digoxin net secretion was increased by 1.6-fold (from 0.54 ± 0.02 to 0.87 ± 0.02 nmol cm$^{-2}$ h$^{-1}$; both $n=14$ paired monolayers, $P<0.05$). For both substrates, the increase in net secretion occurred through a reduction in $J_{b-a}$ and an increase in $J_{a-b}$ relative to the wild-type cells.

Fig. 3A compares the effect of MK-571 (at 50 μM) and the cyclosporin A (at 100 μM) upon the bi-directional transport and net secretion of digoxin (10 μM substrate) in MDCKII-MDR1 cell layers; whereas MK-571 had no significant effect on net secretion ($P>0.05$ vs. control) there was an 82% reduction observed with cyclosporin A ($P<0.05$ vs. control). The marked effect of cyclosporin A was brought about by a reduction in $J_{b-a}$ and an increase in $J_{a-b}$, whereas MK-571 produced an increase in $J_{a-b}$, with no effect on $J_{b-a}$; control $J_{b-a}$ was 1.51 ± 0.05 nmol cm$^{-2}$ h$^{-1}$ vs. 1.50 ± 0.01 nmol cm$^{-2}$ h$^{-1}$ in the presence of 50 μM MK-571 (both $n=4$; $P>0.05$, control vs. MK-571). Fig. 3B also demonstrates that whereas cyclosporin A was capable of increasing cellular digoxin in MDCKII-MDR1 cell layers from both apical and basal surfaces, MK-571 had only a minor effect. Taken together, these data suggest that MK-571 has very little or no effect on MDR1-mediated digoxin secretion. When used in combination, MK-571 and cyclosporin A were no more effective at reducing digoxin $J_{b-a}$ than with cyclosporin A used alone, although a further increase in $J_{a-b}$ was apparent (Fig. 3A). Similarly, the combined treatment of MK-571 and cyclosporin A was no more effective at increasing digoxin accumulation across either cell border than cyclosporin A when used alone (Fig. 3B). Therefore cyclosporin A is capable of inhibiting MDR1-mediated digoxin secretion, whereas MK-571 is not. This contrasts with the data on MK-571 and cyclosporin inhibition of digoxin secretion in Caco-2 epithelia (above).

In order to test directly the participation of the ABC transporter MRP2 in transepithelial secretion of digoxin, we used epithelial layers of MDCKII-*MRP2* cells and compared the ability of these cells to secrete digoxin with non-transfected parental MDCKII cells. Vinblastine is a recognised MRP2 substrate (Evers et al., 1998), and was used as a positive control. Both digoxin and vinblastine were used at 10 μM. Fig. 4 shows that upon MRP2 transfection, whereas

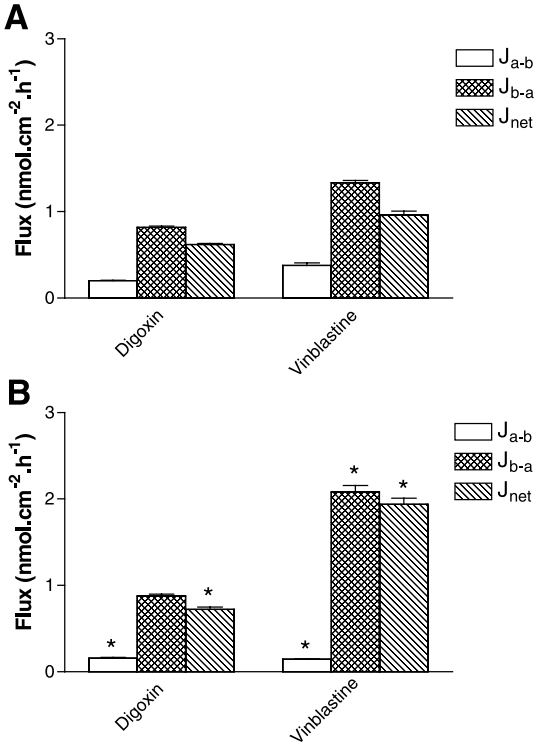


Fig. 4. Comparison of digoxin and vinblastine secretion between MDCKII and MDCKII-*MRP2* epithelial layers. Transepithelial [$^3$H]digoxin and [$^3$H]vinblastine fluxes (both 10 μM) were measured in the apical-to-basal ($J_{a-b}$) and basal-to-apical ($J_{b-a}$) directions in adjacent paired monolayers. Net secretory flux ($J_{net}$)=$J_{b-a}-J_{a-b}$. (A) Parental MDCKII cell monolayers. (B) MDCKII-*MRP2* monolayers. Data are mean ± S.E.M. of six epithelial layers or pairs per column. *$P<0.05$ significantly different from MDCKII wild type control layers.

the absorption of digoxin was significantly reduced, the increase in basal-to-apical transport was not significant. Overall this resulted in statistically significant increase in net secretion of digoxin; however, this was only a 1.2-fold increase, and was modest compared to the increase in secretion observed for vinblastine (2-fold; Fig. 4).

## 4. Discussion

The use of MDR1 (human) or Mdr1 (rodent) transfected model systems such as MDCKII or LLC-$PK_1$ cells has confirmed digoxin as an MDR1/Mdr1 substrate (Schinkel et al., 1995, 1996; present study). Furthermore, the use of mice in which *Mdr1a*, *Mdr1b*, or both genes have been disrupted, has allowed a valuable assessment as to the in vivo importance of mdr transporters in the tissue distribution and pharmacokinetics of numerous drugs, including digoxin (Schinkel et al., 1995, 1997; Mayer et al., 1996). In the double knockout *Mdr1a/b* (−/−) animals, [$^3$H]digoxin accumulation was increased in certain tissues where Mdr1a/b are normally expressed, including the brain, ovaries, and lymph nodes (Schinkel et al., 1997). Intestinal secretion of [$^3$H]digoxin was reduced in both *Mdr1a* (−/−) and the double knockout *Mdr1a/b* (−/−) animals to a similar degree, confirming the importance of mdr1a in mouse intestine. Interestingly, the urinary excretion of digoxin was unchanged in the *Mdr1a/b* (−/−) mice, and biliary clearance was not reduced as markedly as expected, which suggests that the kidney and liver express alternative transport mechanisms to maintain digoxin secretion in these tissues. Do similar mechanisms play a role in human intestinal cells?

In addition to MDR1, digoxin secretion by Caco-2 monolayers also appears to be mediated by an MK-571-sensitive mechanism, although the identity of this transporter is at present unknown. Indeed, the ability of discriminating between the contribution of individual ABC transporters to digoxin secretion in Caco-2 cells depends upon the availability of specific inhibitors. MK-571 is widely used as an MRP-selective inhibitor. For example, Chen et al. (1999) studied the action of various multidrug-reversing agents upon apical MRP2 in transfected LLC-$PK_1$ cells, and found that for inhibition of ATP-driven vesicular Leukotriene $C_4$ uptake, MK-571 was an effective inhibitor of MRP2, with a $K_i$ value of 13 μM. Similar data were reported for MK-571 inhibition of estradiol-17-β-D-glucuronide uptake into membrane vesicles from rabbit MRP2 transfected insect cells (van Aubel et al., 1998). For inhibition of MRP1-mediated daunorubicin transport by MK-571, the $IC_{50}$ was just 0.4 μM (Renes et al., 1999). These data are consistent with effective MRP inhibition. The present data showed that MK-571 did not affect MDR1-mediated basal-to-apical transport across MDCKII-MDR1 cell monolayers, although the absorptive flux was increased. This indicates that any effect of MK-571 on MDR1-mediated transport is likely to be minimal. Furthermore, it is a possibility that MK-571 interacts with alternative mechanisms, distinct from ABC transporters. In Caco-2 cell layers, MK-571-mediated inhibition of digoxin secretion comprised a substantial fraction (0.51) of total secretion and also occurs within the dose-range expected for inhibition of MRP2.

The inhibitory monoclonal antibody MRK16 is directed against an external epitope of the P-glycoprotein molecule and was used here to investigate whether digoxin transport was consistent with MDR1-mediated secretion in Caco-2 cells (Hunter et al., 1993b). The differential action of MRK16 on the basal-to-apical fluxes of vinblastine and digoxin points to a difference in the mechanism of secretion. Two explanations are possible. First, a recent study has postulated that MDR1 may have at least four distinct binding sites for substrates and modulators (Martin et al., 2000). MRK16 could then inhibit binding and transport of vinblastine but not digoxin. However, this is unlikely since Ushigome et al. (2000) have shown that MRK16 significantly increases MDR1-mediated digoxin uptake by cultured human placental choriocarcinoma epithelial cells. The second explanation is that multiple ABC transporters with overlapping substrate specificities may be responsible. For vinblastine secretion, both MDR1 and MRP2 contribute to this transport in Caco-2 cell layers (Evers et al., 1998; present data). For digoxin secretion, the MRK16 data suggest minimal involvement of MDR1. However this cannot be the case since digoxin secretion is enhanced in MDCKII-*MDR1* monolayers, and cyclosporin A reduced net secretion in both Caco-2 and MDCKII-*MDR1* monolayers. Therefore the most likely explanation for the lack of MRK16 inhibition is that basal-to-apical flux is maintained via a non-MDR1 transporter after prolonged incubation with the antibody.

Is MRP2 the alternative pathway for digoxin secretion? Although vinblastine secretion was enhanced in MDCKII-*MRP2* layers, there was only a modest (1.2-fold) increase in digoxin secretion, which occurred principally through a reduction in the absorptive flux. This suggests that MRP2 is unlikely to contribute to digoxin secretion. Our previous studies in Caco-2 cells (Cavet et al., 1996) have used 1-chloro-2,4-dinitrobenzene (CDNB) to test for the involvement of the MRP family in digoxin secretion by Caco-2 cells; the lack of inhibition suggested no involvement. Furthermore, using Caco-2 cells, Stephens et al. (2001) also found that MK-571 significantly reduced net secretion of digoxin. However, in mouse ileum, which also functionally expresses MRP2 at the apical membrane, MK-571 had no inhibitory effect (Stephens et al., 2001, 2002). The mdr1 inhibitor quinidine, on the other hand, completely abolishes digoxin secretion in mouse intestine (Stephens et al., 2002). This suggests that in mice, mdr1 contributes exclusively to digoxin secretion by the intestine, which is consistent with the data from *Mdr1a* (−/−) mice (Schinkel et al., 1997; Stephens et al., 2002). However, in humans, this may not be the case; the present data from Caco-2 cells suggest that in

addition to MDR1, it is likely that digoxin is secreted by a mechanism distinct from MRP2, but sensitive to MK-571. Interestingly, a recent study has reported that MK-571 inhibited the exit of fluo-3 and fura red across the apical membrane of the human colonic cell line HT29 (clone 19A) (Abrahamse and Rechkemmer, 2001). Given that MRP2 is not expressed in HT29 cells (Abrahamse and Rechkemmer, 2001), this transport system must be distinct from MRP2. The identity and specific characteristics of this transporter remain to be elucidated.

It is becoming increasingly evident that epithelial surfaces include multiple ABC transporters to maintain physiological function. For instance, the canalicular (apical) membrane of hepatocytes expresses multiple ABC transporters including P-glycoprotein (MDR1), MDR3 (the phosphatidylcholine translocator), sister of P-glycoprotein (SPGP; or bile salt export pump, BSEP), and MRP2 for certain bile acids and non-bile acid organic anions (Kipp et al., 2001; Ortiz et al., 1999). In human intestine and in Caco-2 cells, it is clear that both MDR1 and MRP2 are expressed (Hirohashi et al., 2000; Stephens et al., 2001; Taipalensuu et al., 2001; Lowes and Simmons, 2002), though variation is evident (Gutmann et al., 1999). Recently it has been reported that the ABC-half transporter BCRP (breast cancer resistance protein; Doyle et al., 1998) is expressed at the apical membrane of both small intestine and colon (Maliepaard et al., 2001), and is also expressed in Caco-2 cells, though to a considerably lesser extent (Taipalensuu et al., 2001). The full extent to which BCRP contributes to intestinal drug disposition remains to be determined, although it might well be significant (Taipalensuu et al., 2001). Whereas intestinal secretion of digoxin in mice may result solely from mdr1 proteins (Schinkel et al., 1997), the current data indicate that this is likely not to be the case for human intestine. The involvement of certain transport proteins, such as MRP1 and MRP3, in the secretion of solutes in the basal-to-apical direction across intestinal epithelia is precluded since these proteins are widely established as basolateral export proteins.

In summary, whereas MDR1 plays a significant role in the secretion of digoxin across human intestinal epithelial Caco-2 cells, there is evidence to suggest that other secretory proteins may also contribute to digoxin secretion.

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