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ORIGINAL ARTICLE

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P-glycoprotein-mediated transport of digitoxin, α-methyldigoxin and β-acetyldigoxin

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Abstract Digoxin is a drug with a narrow therapeutic index, which is substrate of the ATP-dependent efflux pump P-glycoprotein. Increased or decreased digoxin plasma concentrations occur in humans due to inhibition or induction of this drug transporter in organs with excretory function such as small intestine, liver and kidneys. Whereas particle size, dissolution rate and lipophilic properties have been identified as determinants for absorption of digitalis glycosides, little is known about P-glycoprotein transport characteristics of digitalis glycosides such as digitoxin, α-methyldigoxin, β-acetyldigoxin and ouabain. Using polarized P-glycoprotein-expressing cell lines we therefore studied whether these compounds are substrates of P-glycoprotein.

Polarized transport of digitalis glycosides was assessed in P-glycoprotein-expressing Caco-2 and L-MDR1 cells (LLC-PK1 cells stably transfected with the human MDR1 P-glycoprotein). Inhibition of P-glycoprotein-mediated transport of these compounds in Caco-2 cells was determined using the cyclosporine analogue PSC-833 (valspodar) as inhibitor of P-glycoprotein.

No polarized transport was observed for ouabain. However, basal-to-apical transport of digitoxin, α-methyldigoxin and β-acetyldigoxin was greater than apical-to-basal transport in Caco-2 and L-MDR1 cells. In Caco-2 cells net transport rates of these compounds were similar to those of digoxin (digoxin: 16.0±4.4%, digitoxin: 15.0±3.3%, β-acetyldigoxin: 16.2±1.6%, α-methyldigoxin: 13.5±4.8%). Furthermore, polarized transport of these compounds could be completely inhibited by 1 μM PSC-833. In summary, these data provide evidence that not only digoxin, but also digitoxin, α-methyldigoxin and β-acetyldigoxin are substrates of P-glycoprotein.

Keywords Digitalis glycosides · Digoxin · P-glycoprotein · Caco-2

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Introduction

Cardiac glycosides are commonly used for treatment of supraventricular arrhythmias and cardiac failure. They comprise a family of drugs with narrow therapeutic index. Elevated digitalis plasma levels frequently result in symptoms of toxicity. Increased plasma levels of digoxin, digitoxin and digoxin derivatives have been described during concomitant treatment with several other drugs including verapamil, nifedipine, nitrendipine, propafenone, amiodarone, quinidine, cyclosporin and itroconazole (Belz et al. 1983; Bigger and Leahey 1982; De Cesaris et al. 1983a, 1983b; Laer et al. 1998; Nademanee et al. 1984; Partanen et al. 1996; Pedersen et al. 1981; Reiffel et al. 1979; Robieux et al. 1992; Sphakianaki et al. 1992).

Transport of digoxin by the ATP-dependent efflux transporter P-glycoprotein has been extensively studied and changes in P-glycoprotein function have been shown to have considerable impact on plasma levels and toxicity of digoxin. Recent studies have attributed drug interactions during treatment with digoxin to an inhibition of P-glycoprotein (Fromm et al. 1999; Woodland et al. 1997). Furthermore it has been shown that a newly described mutation of the *MDR1* gene affects intestinal P-glycopro-

tein expression and digoxin disposition after oral administration (Hoffmeyer et al. 2000).

The *MDR1* gene (ABCB1) product P-glycoprotein is a member of the ABC-transporter family (ATP-Binding Cassette-family). P-glycoprotein is a drug transporter with wide substrate specificity, located mainly in tissues with excretory function (e.g. brush border membrane of proximal tubular cells in kidneys, brush border membrane of enterocytes in intestine, canalicular membrane of hepatocytes, capillary endothelial cells of the brain). There is evidence that P-glycoprotein has considerable impact on bioavailability, tissue concentrations and pharmacodynamic effects of drugs (Fromm et al. 1999; Kim et al. 1998; Schinkel et al. 1995). Due to its location in tissues with excretory function, inhibition of P-glycoprotein activity results in reduced drug elimination via the bile or the urine. On the other hand, induction of intestinal P-glycoprotein expression has been shown to determine digoxin plasma concentration after oral administration (Greiner et al. 1999).

In the 1970s bioavailability of digoxin was relatively low (approximately 60%; Kuhlmann 1982). Therefore more lipophilic digoxin derivatives were developed by methylation and acetylation in order to improve bioavailability. Differences in crystal size, in vitro dissolution rate and lipophilicity have been shown to contribute to different bioavailability of digitalis glycosides in humans (Peters 1982). However, to our knowledge it is not known whether other currently used digitalis glycosides (digitoxin, α-methyldigoxin, β-acetyldigoxin) are also substrates of P-glycoprotein and whether different affinity of these compounds to P-glycoprotein might contribute to differences in pharmacokinetic properties of these substances.

In Germany, digoxin use has been almost completely replaced by these other cardiac glycosides. According to recent prescription statistics, digoxin derivatives (acetyldigoxin, methyldigoxin) account for about 52% of glycoside use in Germany followed by digitoxin (40%) whereas digoxin accounts for only 7% of total glycoside treatment (Scholz 1997). All digitalis glycosides share the same mechanism of action, but they differ in their pharmacokinetic profiles (Flasch 1975; Fricke 1982; Greeff et al. 1977; Lauterbach 1977; Rietbrock et al. 1976). Additionally, differences in tissue distribution have been described in humans, with brain accumulation of methyldigoxin being higher than that of digoxin (Bodem et al. 1977; Haasis and Larbig 1976). After partial demethylation and deacetylation, digoxin derivatives undergo renal elimination. For digitoxin elimination of the parent compound or metabolites occurs via bile and urine. Elevated plasma levels of digitoxin and digoxin derivatives have been described under concomitant use with other drugs (De Cesaris et al. 1983a, 1983b; Laer et al. 1998) which are known to inhibit P-glycoprotein function (Arboix et al. 1997; Cavet et al. 1996; Mickisch et al. 1991; van der Graaf et al. 1991). The mechanism of these interactions has not yet been identified, but an involvement of P-glycoprotein might be suspected.

Using P-glycoprotein-expressing cell lines (Caco-2, L-MDR1) and the P-glycoprotein inhibitor PSC-833 (valspodar) we therefore studied whether the cardiac glycosides digitoxin, α-methyldigoxin, β-acetyldigoxin and ouabain are substrates of P-glycoprotein.

Materials and methods

Materials

[^{3}H]digoxin (19 Ci/mmol), [^{3}H]digitoxin (15.9 Ci/mmol) and [^{3}H]ouabain (16.5 Ci/mmol) were supplied by NEN Life Science Products (Boston, Mass., USA). Unlabeled α-methyldigoxin was obtained from Boehringer Mannheim (Mannheim, Germany) and β-acetyldigoxin was a gift from the Arzneimittelwerk Dresden (Dresden, Germany). Unlabeled digoxin was purchased from Sigma (Germany). [$^2H_3$]digoxin was synthesized according to Haberland and Maerten (Haberland and Maerten 1969). PSC-833 (valspodar) was a generous gift from Novartis (Basel, Switzerland).

Transport in cultured LLC-PK1, L-MDR1 and Caco-2-cells

Transport studies. Transport was studied using Caco-2 (passage nos. 33–52), L-MDR1 and LLC-PK1 cells (passage nos. 23–40 and 20–45, respectively). Caco-2 cells are a human colon carcinoma cell line, LLC-PK1 cells are porcine kidney epithelial cells and L-MDR1 cells are LLC-PK1 cells stably transfected with human MDR1 cDNA. When grown as a monolayer on semiporous filters, these cells become polarized and P-glycoprotein is expressed in Caco-2 and L-MDR1 cells on their apical surface, allowing study of vectorial transcellular transport, i.e. basal-to-apical and apical-to-basal transport (Fromm et al. 1999; Kim et al. 1998).

L-MDR1 and LLC-PK1 cells were kindly provided by Dr. A.H. Schinkel (Netherlands Cancer Institute, The Netherlands) and Caco-2 cells were obtained from the American Type Culture Collection. Cells were plated on Transwell filters (Costar) and grown under identical conditions as previously described (Fromm et al. 1999; Kim et al. 1998). Transport experiments were performed on day 7 after plating. About 1 h prior to the start of the transport experiment, the medium in each compartment was replaced by serum-free medium (Optimem, Gibco BRL). For transport experiments the medium in each compartment was then replaced with 800 µl serum-free medium with addition of the drug (5 µM) on the basal or the apical side of the monolayer. The amount of drug appearing in the opposite compartment (basal or apical) after 1, 2, 3 and 4 h was measured in 50-µl aliquots. Drug transport was calculated as percent of the amount added. Net basal-to-apical transport was calculated after 4 h by subtracting the basal-to-apical from the apical-to-basal transport rate. Moreover, net apparent permeability coefficients ($P_{app/net}$) were calculated by subtracting the apparent permeability coefficients (P_{app}) from the initial basal-to-apical and apical-to-basal transport rates. P_{app} was determined according to

$$P_{app} = dQ/dt * 1/(A * C_0)[cm/s] \quad (1)$$

where dQ/dt (µmol/s) is the initial transport rate, C_0 (µmol/cm^3) the initial concentration in the donor chamber and A (cm^2) the surface area of the monolayer (Artursson and Karlsson 1991).

Inhibition experiments. Inhibition of P-glycoprotein-mediated transport of digoxin, digitoxin, α-methyldigoxin and β-acetyldigoxin across confluent Caco-2 cell monolayers was determined in a similar manner after addition of the cyclosporin analogue PSC-833 (1 µM) to both the apical and the basal compartment. Complete inhibition of P-glycoprotein-mediated transport would be expected to result in the loss of basal-to-apical vs. apical-to-basal transport difference. Digoxin transport was determined for each plate (12 wells) in the absence of any inhibitor as positive control (2 wells/plate). Experiments were conducted only with wells that

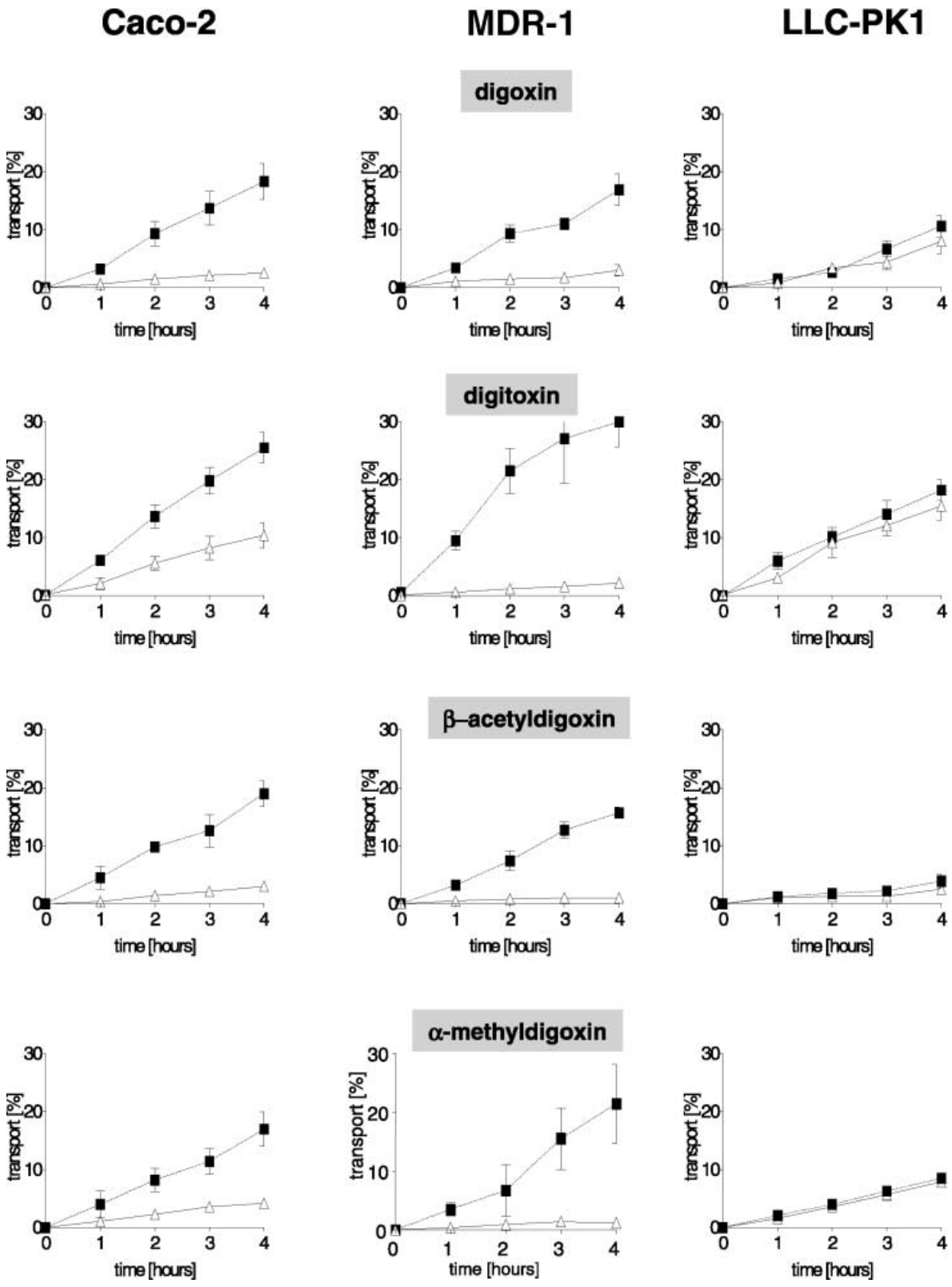

Fig. 1 Transepithelial transport of digoxin, digitoxin, α-methyldigoxin and βacetyldigoxin across Caco-2, L-MDR1 and LLC-PK1 monolayers (*filled squares* translocation from the basal to the apical compartment, *open triangles* translocation from the apical to the basal compartment). Data are means ± 1 standard deviation from three or more experiments

Table 1 Net transport (T_{net}) and net apparent permeability coefficient ($P_{app/net}$ [cm/s] $\times 10^{-6}$) of digoxin, digitoxin, β-acetyldigoxin and α-methyldigoxin in Caco-2, L-MDR1 and LLC-PK1 cells (means ± SD)

	Caco-2		L-MDR1		LLC-PK1	
	T_{net}	$P_{app/net}$	T_{net}	$P_{app/net}$	T_{net}	$P_{app/net}$
Digoxin	16.0±4.4	6.4±2.7	11.7±3.6	6.4±2.2*	3.3±1.1	2.0±0.9
Digitoxin	15.0±3.3	11 ±3.2	30.0±7.3	24.6±5.6	2.7±2.5	4.4±1.2
β-Acetyldigoxin	16.2±1.6	8.8±3.4	14.7±0.7	7.7±0.7*	1.5±1.0	0.6±0.1
α-Methyldigoxin	13.5±4.8	8.2±6.4	20.2±6.6	8.3±2.9*	0.6±0.8	1.3±0.9

*P<0.001 digoxin, β-acetyldigoxin and α-methyldigoxin vs. digitoxin

had a transepithelial resistance of >200 Ω after correction for the resistance obtained in control blank wells.

Drug analyses

Aliquots containing radiolabeled ouabain, digoxin and digitoxin were analyzed by liquid scintillation counting (Beckman, Germany) after the addition of 5 ml Aqua safe 300 plus (Zinsser Analytic, Frankfurt, Germany).

To quantitate α-methyldigoxin, β-acetyldigoxin and digoxin in transport studies with unlabeled compounds, an LC-MSD 1100 system (Agilent Technologies, Waldbronn, Germany) was used. To 45 µl of serum from either the apical or the basal side of the cell monolayer 6 µl of a solution of [$^2H_3$]digoxin (10 pmol/µl in acetonitrile) as an internal standard was added. A 10-µl aliquot of the sample was injected. Separation was performed on a reversed-phase column (HyPURITY C18; 5 µM, 50×2.1 mm) using a gradient from 10% to 40% acetonitrile (+5 mM ammonium acetate) in 5 mM ammonium acetate in water. Electrospray ionization produced sodium adducts which were monitored in the single ion mode at m/z=803.4, 806.4, 817.5 and 845.5 for digoxin, [$^2H_3$]digoxin, α-methyldigoxin and β-acetyldigoxin, respectively. Calibration curves were linear from 0.1 µM to 10 µM. Quality controls were routinely assayed at 0.1, 1 and 5 µM with coefficients of variation and bias being less than 10%.

Statistical analysis

All data are presented as means ± 1 standard deviation. Mean values were calculated from at least three experiments conducted on different days. Differences in net apparent permeability coefficients were tested for significance by ANOVA with subsequent Student-Newman-Keuls post-hoc tests (Instat 1997; GraphPad Software, San Diego, Calif., USA). A value of P<0.05 was required for statistical significance.

Results

Methyldigoxin was not demethylated during the transport experiments in Caco-2 and L-MDR1 cells. During basal-to-apical translocation 18.5±2.0% and 23.7±1.3% of acetyldigoxin were deacetylated to digoxin in L-MDR1 and Caco-2 cells, respectively (during apical-to-basal translocation: 47.2±0.6% and 36.5±3.2%, respectively). Transport rates were calculated only for the unchanged fraction of digoxin derivatives.

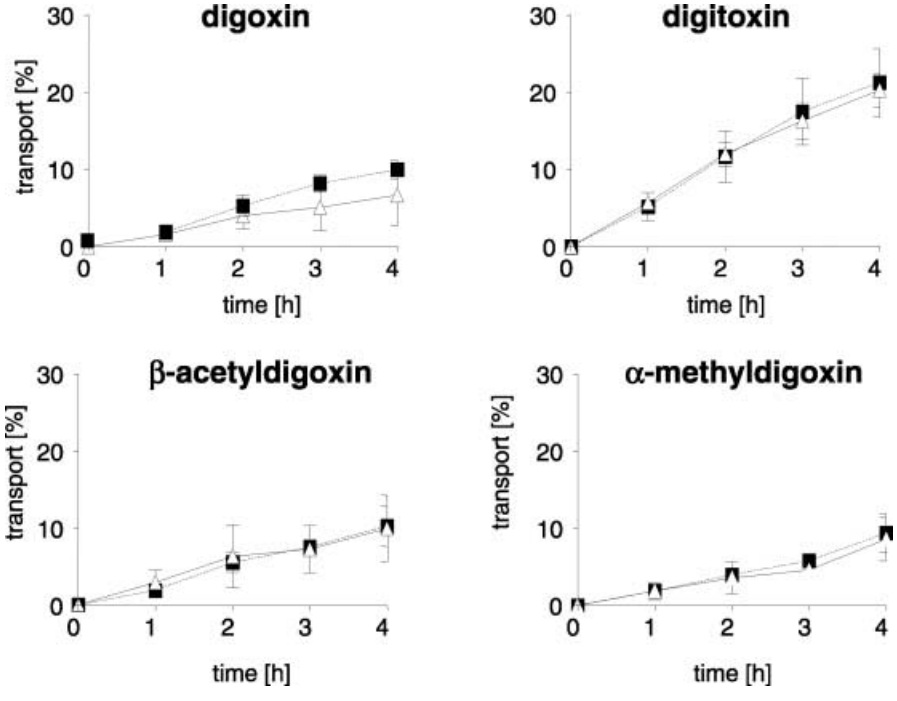

Fig. 2 Inhibition of P-glycoprotein-mediated transport of digoxin, digitoxin, α-methyldigoxin and β-acetyldigoxin in Caco-2 cells by the cyclosporin analogue PSC-833 (1 µM). Data are means ± 1 standard deviation from three or more experiments

Transport studies

No polarized ouabain transport was found in Caco-2 and L-MDR1 cells (data not shown). Digoxin, digitoxin, α-methyldigoxin and β-acetyldigoxin cellular translocation was markedly greater when the drug was administered to the basal side of cultured L-MDR1 and Caco-2 cells and its appearance measured on the apical side (basal-to-apical) compared to addition of drugs to the opposite compartment (apical-to-basal; Fig. 1). In LLC-PK1 cells, such an apical-to-basal and basal-to-apical transport difference was absent for all compounds tested. There was no significant difference in net apparent permeability coefficients ($P_{app/net}$) from basal-to-apical between digoxin, digitoxin, α-methyldigoxin and β-acetyldigoxin in Caco-2 cells. However, in L-MDR1 cells net apparent permeability coefficient of digitoxin was significantly higher than those of digoxin, α-methyldigoxin and β-acetyldigoxin ($P<0.001$; Table 1).

Inhibition experiments

With addition of PSC-833 to Caco-2 cells this directional transport difference disappeared for all digitalis glycosides (Fig. 2).

Discussion

The present investigation provides evidence that not only digoxin but also digitoxin and the digoxin derivatives α-methyldigoxin and β-acetyldigoxin are P-glycoprotein substrates. We showed that net basal-to-apical transport of these compounds is greater than apical-to-basal transport in P-glycoprotein expressing L-MDR1 and Caco-2 cells. Transport could be inhibited by the P-glycoprotein inhibitor PSC-833. While basal to apical net transport of digitoxin, α-methyldigoxin and β-acetyldigoxin was not significantly different from digoxin transport in Caco-2 cells, significantly higher transport rates could be found for digitoxin in L-MDR1 cells in comparison to the other compounds.

An active intestinal transport mechanism for cardiac glycosides was first postulated by Lauterbach (1977). Using an in vitro system of isolated human intestinal mucosa epithelium, he observed secretion of different digitalis glycosides from the basal side of the mucosa into the intestinal lumen (Lauterbach 1977). Recently, this transport has been attributed to the function of the ATP-dependent efflux-transporter P-glycoprotein (Cavet et al. 1996; Mayer et al. 1996), since digoxin is transported in polarized P-glycoprotein-expressing cell lines and loss of P-glycoprotein function in P-glycoprotein-deficient mdr1a knockout mice is accompanied by an increase in plasma and tissue levels of digoxin (Schinkel et al. 1995). Other efflux transporters such as rat oatp2, which is highly expressed in brain, liver and kidneys, have been described to be involved in transport of cardiac glycosides (Noe et al. 1997) but until now there are no data available on the impact of this transporter for glycoside disposition in humans.

Recent studies provided compelling evidence that the mechanism underlying the digoxin-quinidine interaction is related to inhibition of P-glycoprotein by quinidine (Fromm et al. 1999). Inhibition of P-glycoprotein function appears to be a common mechanism explaining elevated digoxin concentrations under therapy with multiple other drugs recognized to be P-glycoprotein inhibitors, such as various antiarrhythmic agents, cyclosporin and itroconazole. Moreover, decreased digoxin plasma concentrations in humans during concomitant treatment with rifampin could be attributed to induction of intestinal P-glycoprotein (Greiner et al. 1999). Finally, it has recently been shown that in addition to the environmental factors mentioned above mutations of the *MDR1* gene affect intestinal P-glycoprotein expression and digoxin disposition after oral administration (Hoffmeyer et al. 2000).

The digoxin derivatives methyldigoxin and acetyldigoxin were developed to increase intestinal absorption and bioavailability of digitalis glycosides, thereby reducing interindividual variation in drug absorption and the risk of drug toxicity (Johnson et al. 1978). Digitoxin, β-acetyldigoxin and α-methyldigoxin are almost completely absorbed after oral administration (Fachinformation Coramedan 1999; Fachinformation Lanitop 1994; Lauterbach 1977). Increased intestinal absorption has been attributed to increased lipophilicity of these compounds. According to octanol-water distribution coefficients, lipophilicity of β-methyldigoxin (54.6), digitoxin (70.0), and β-acetyldigoxin (93.0) is considerably higher than that of digoxin (18.2; Cohnen et al. 1978). In humans, acetyldigoxin is almost completely deacetylated to digoxin during intestinal absorption (Lauterbach 1977). Thus, whereas intestinal P-glycoprotein located in the brush border membrane of the enterocytes might to some extent alter absorption of β-acetyldigoxin, drug interactions after administration of β-acetyldigoxin with P-glycoprotein-inhibiting drugs will be due to impaired elimination of digoxin. We determined the β-acetyldigoxin fraction being deacetylated in Caco-2 and L-MDR1 cells and transport rates were calculated only for unchanged β-acetyldigoxin. In both Caco-2 and L-MDR1 cells, approximately 75% and 55% of the initially applied β-acetyldigoxin was transported as unchanged drug from basal-to-apical and apical-to-basal, respectively. α-Methyldigoxin was not demethylated during transport experiments. This is in accordance to human data, where demethylation of methyldigoxin occurs mainly in the liver (Rietbrock et al. 1976). Our data provide evidence that differences in drug absorption and bioavailability of digitalis glycosides can not be explained by a lower affinity of digitoxin and digoxin derivatives to P-glycoprotein because the transport rates of the studied glycosides were comparable with those of digoxin in Caco-2 cells.

On the other hand, our data point to a new mechanism to explain drug interactions observed under therapy with digitoxin and methyldigoxin. There are several publica-

tions describing severe drug interactions under therapy with digitoxin and digoxin derivatives. Digitoxin and methyldigoxin plasma levels were found to be elevated under therapy with other antiarrhythmic drugs, such as amiodarone, verapamil, diltiazem and propafenone (De Cesaris et al. 1983a, 1983b; Laer et al. 1998; Sphakianaki et al. 1992). The underlying mechanism of these interactions has not yet been investigated. Displacement of tissue-bond digitoxin by amiodarone and inhibition of the cytochrome P-450 system have been proposed as possible mechanisms, as cytochrome P450 3A4 is involved in the metabolism of digitoxin in the rat (Eberhart et al. 1992). Elevated plasma levels of digitoxin under concomitant use of verapamil and diltiazem were attributed to inhibition of hepatic digitoxin metabolism by these drugs.

There are several lines of evidence that P-glycoprotein might play an important role in the observed drug interaction with digitoxin and digoxin derivatives. First, in vitro and in vivo studies have provided evidence that all drugs causing increased digitalis glycoside concentrations are known P-glycoprotein inhibitors. For example, verapamil and propafenone both inhibit digoxin-transport in P-glycoprotein-expressing cell lines (Cavet et al. 1996; Woodland et al. 1997). In vitro studies in renal cell carcinoma revealed that calcium antagonists such as verapamil showed the strongest reversal of chemoresistance, but derivatives of the benzothiazepine type (diltiazem) acted similarly and reached about 70% of the verapamil activity (Mickisch et al. 1991). Animal models have provided evidence of the P-glycoprotein-inhibiting properties of amiodarone (Arboix et al. 1997). Furthermore, verapamil and amiodarone have been used in vivo as multidrug resistance-reversing agents in cancer chemotherapy (Eichelbaum et al. 1993; van der Graaf et al. 1991). Second, although cytochrome P450 enzymes are involved in digitoxin metabolism (Eberhart et al. 1992), they cannot account for drug interactions observed under therapy with digoxin derivatives, as in humans these drugs are not metabolized to a major extent by cytochrome P450. Finally, direct evidence for a link between P-glycoprotein and basal-to-apical transport of digitoxin and digoxin derivatives can stem from the finding of complete transport inhibition for digitoxin, α-methyldigoxin and β-acetyldigoxin by PSC-833, which has been developed as a P-glycoprotein inhibitor. The inhibition of transcellular P-glycoprotein-mediated efflux transport by PSC-833 in Caco-2 cells, which have many characteristics of intestinal epithelial cells, provides a likely explanation for the observed increase in plasma levels of digitoxin and digoxin derivatives under concomitant therapy with P-glycoprotein inhibitors. Thus, inhibition of P-glycoprotein-mediated basal-to-apical transport may not only serve to explain digoxin drug interactions, but may be applied to other cardiac glycosides used in clinical practice.

In summary, our data provide evidence that digitoxin and the digoxin derivatives α-methyldigoxin and β-acetyldigoxin are P-glycoprotein substrates. Differences in intestinal absorption and tissue distribution of cardiac glycosides are unlikely to be due to differences in P-glycoprotein-dependent efflux. Moreover, changes in P-glycoprotein function due to concomitant use of P-glycoprotein-inhibiting or -inducing drugs is likely to be the underlying mechanism of drug interactions observed during therapy with these cardiac glycosides.

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