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Substantial excretion of digoxin via the intestinal mucosa and prevention of long-term digoxin accumulation in the brain by the mdr1a P-glycoprotein

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1 We have used mice with a disrupted *mdr1a* P-glycoprotein gene (*mdr1a* (−/−) mice) to study the role of P-glycoprotein in the pharmacokinetics of digoxin, a model P-glycoprotein substrate.

2 [^{3}H]-digoxin at a dose of 0.2 mg kg^{-1} was administered as a single i.v. or oral bolus injection. We focussed on intestinal mucosa and brain endothelial cells, two major pharmacological barriers, as the mdr1a P-glycoprotein is the only P-glycoprotein normally present in these tissues.

3 Predominant faecal excretion of [^{3}H]-digoxin in wild-type mice shifted towards predominantly urinary excretion in *mdr1a* (−/−) mice.

4 After interruption of the biliary excretion into the intestine, we found a substantial excretion of [^{3}H]-digoxin via the gut mucosa in wild-type mice (16% of administered dose over 90 min). This was only 2% in *mdr1a* (−/−) mice. Biliary excretion of [^{3}H]-digoxin was not dramatically decreased (24% in wild-type mice versus 16% in *mdr1a* (−/−) mice).

5 After a single bolus injection, brain levels of [^{3}H]-digoxin in wild-type mice remained very low, whereas in *mdr1a* (−/−) mice these levels continuously increased over a period of 3 days, resulting in a ~200 fold higher concentration than in wild-type mice.

6 These data demonstrate the *in vivo* contribution of intestinal P-glycoprotein to direct elimination of [^{3}H]-digoxin from the systemic circulation and to the pattern of [^{3}H]-digoxin disposition, and they underline the importance of P-glycoprotein for the blood-brain barrier.

Keywords: Multidrug resistance; drug disposition; blood-brain barrier; knockout mice; enterohepatic circulation; intestinal mucosa; P-glycoprotein

Introduction

Resistance to chemotherapy is a major problem in the treatment of cancer. P-glycoprotein (P-gp), a 170 kDa transmembrane protein found in a variety of tumor cell lines (Juliano & Ling, 1976), probably contributes to resistance, e.g. in osteosarcoma, leukaemias and neuroblastoma (Bourhis *et al.*, 1989; Bellamy *et al.*, 1990; Marie *et al.*, 1991; Baldini *et al.*, 1995). *In vitro* studies showed that P-gp can transport a wide range of hydrophobic, amphipathic compounds (reviewed by Gottesman & Pastan, 1993). P-gp protects cells from damage by transporting these potentially cytotoxic agents, which include carcinostatic drugs, such as anthracyclines, Vinca alkaloids, etoposide and paclitaxel, out of the cell. A number of compounds with relatively low toxicity can inhibit drug transport by P-gp (Tsuruo *et al.*, 1981; Ford & Hait, 1990), and these so-called reversal agents are being tested in clinical trials to increase the response to chemotherapy of treatment-resistant tumours, thus far with limited success (Sikic *et al.*, 1994; Wishart *et al.*, 1994; Pinedo & Giaccone, 1995; Dalton *et al.*, 1995; Wilson *et al.*, 1995). As toxicity of the early reversal agents did not allow efficient blockade of P-gp (Yuen & Sikic, 1994), more efficient and less toxic reversal agents, such as the cyclosporin A analogue, SDZ PSC 833, have been developed and are entering clinical trials (Twentyman & Bleehen, 1991; Sikic, 1995). The use of these effective new reversal agents requires more knowledge of the physiological function of P-gp and of the pharmacological consequences of blocking this function in normal organs. In human subjects, high levels of the MDR1 P-gp are found in the apical membrane of the intestinal epithelium, the proximal tubules of the kidney, in the biliary canalicular membrane of the hepatocytes, and in the endothelial cells of the brain at the position of the blood-brain barrier (Thiebaut *et al.*, 1987; Cordon-Cardo *et al.*, 1989). Mice have two multidrug resistance genes (*mdr1a* and *mdr1b*), which together may fulfil the same function as the *MDR1* gene in humans alone (reviewed by Borst & Schinkel, 1996).

To get a better understanding of the physiological function of P-gp and its influence on the pharmacokinetics of transported drugs, we have generated mice with a disrupted *mdr1a* gene (*mdr1a* (−/−) mice) (Schinkel *et al.*, 1994). Under laboratory conditions the *mdr1a* (−/−) mice are viable and show no major pathology. Whereas the mdr1a P-gp is abundant in the intestinal epithelium and the brain endothelial cells of wild-type mice, *mdr1a* (−/−) mice do not contain any detectable P-gp in these tissues. Previous experiments with *mdr1a* (−/−) mice showed that mdr1a P-gp is an important element of the blood-brain barrier. We found highly increased brain concentrations and altered tissue distribution of a range of drugs transported by P-gp, including ivermectin, vinblastine, digoxin, cyclosporin A and dexamethasone (Schinkel *et al.*, 1994, 1995). P-gp-mediated digoxin transport was also demonstrated *in vitro* in multidrug-resistant Chinese hamster ovary cells (de Lannoy & Silverman, 1992), or a porcine kidney epithelial cell line, transfected with the human MDR1 cDNA (Tanigawara *et al.*, 1992; Ito *et al.*, 1993; Okamura *et al.*,

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1993), or mouse mdr1a cDNA (Schinkel *et al.*, 1995). There is also *in vivo* evidence that digoxin is secreted by the tubular epithelium, in addition to its glomerular filtration (Steiness, 1974; de Lannoy *et al.*, 1992; Hori *et al.*, 1993). However, the involvement of P-gp in this urinary excretion process remains to be determined.

From these experiments, [^{3}H]-digoxin seemed to be an interesting model substrate to study further the function of P-gp in drug pharmacokinetics using *mdr1a* (−/−) mice. An additional reason for choosing digoxin for a more detailed analysis was the known clinical interaction of digoxin with drugs such as verapamil, quinidine and amiodarone, which are effective reversal agents for P-gp (Ford & Hait, 1990). These compounds cause elevated digoxin plasma levels in human subjects when given in combination with this cardiac glycoside (Hager *et al.*, 1979; Schwartz *et al.*, 1980; Moysey *et al.*, 1981). Because of the absence of drug transporting P-gp from the apical membrane of the intestinal epithelium and the endothelial cells of the brain of *mdr1a* (−/−) mice, we focussed this study on excretion of [^{3}H]-digoxin and on drug distribution in the brain.

Methods

Drug distribution and excretion experiments

Female mice of a mixed genetic background (on average 50% FVB, 50% 129/Ola) between 10–14 weeks of age were used. Procedures involving experiments with animals and their care were carried out according to institutional guidelines complying with Dutch national law. The unlabelled digoxin stock (2 mg ml^{-1} in 2.6 M 1,2-propanediol, 8.7 M ethanol, 0.1 M glucose) was diluted in 0.3 M glucose to 0.04 mg ml^{-1} or 0.01 mg ml^{-1} (for 0.05 mg kg^{-1}) for intravenous (i.v.) injection and 0.02 mg ml^{-1} for oral administration. After adding the radiolabelled digoxin (1 μCi 30 g^{-1} body weight), 5 μl per gram body weight (0.2 mg kg^{-1}) was injected for i.v. administration and 10 μl per gram body weight (0.2 mg kg^{-1}) for oral administration. After light anaesthesia using diethylether, drug solution was injected into the tail vein (i.v.), or injected by gavage into the stomach using a blunt-ended needle (oral). Mice were killed and tissues were collected at fixed time points after drug administration: 1 h, 4 h, 24 h and 72 h for both i.v. and oral administration of [^{3}H]-digoxin. An 8 h and 120 h time point was added for the i.v. administration of [^{3}H]-digoxin. A dose of 0.05 mg kg^{-1} was administered i.v. for a 72 h time point. Each experiment was carried out with 3 wild-type and 3 *mdr1a* (−/−) mice. All samples were collected in plastic vials. The gallbladder was processed together with its biliary content. To separate the contents of the intestine from the intestinal wall, they were gently pressed out of the respective gut segments with the rounded edge of a forceps; during this procedure the gut segment remained inside-in. To collect faeces and urine, mice were kept in metabolic cages. The excreted faeces, collected in these cages, were combined in one fraction with the contents of the colon. After weighing, tissues and contents of the intestine were homogenized in 4% (w/v) bovine serum albumin. Plasma was prepared by orbital bleeding, collection in eppendorf tubes containing 4 μl heparin (5000 iu ml^{-1}), and centrifugation; 200 μl aliquots (100 μl for plasma, 25 μl for urine and bile) were transferred to 4 ml Ultima Gold counting fluid (Packard, Meriden, CT, U.S.A.) and radioactivity was measured by liquid scintillation counting.

Gallbladder cannulation experiments

Mice were anaesthetized with a combination of Hypnorm (fentanyl 0.2 mg ml^{-1}, fluanisone 10 mg ml^{-1}) and Dormicum (midazolam 5 mg ml^{-1}). It was used as a mixture of 2 parts 0.3 M glucose, 1 part Hypnorm and 1 part Dormicum. The volume of the anaesthetic injected intraperitoneally was 7 μl per g body weight. A sufficient depth of anaesthesia was assessed by negative pain-reflexes and a careful supervision of breathing frequency and noise-sensitivity of the anaesthetized mouse during the experiment. To prevent mice from developing hypothermia, they were put on an electric heating pad, maintaining a rectal temperature of 36–37°C. An infrared lamp was used if additional heating was necessary. After opening of the abdominal cavity and distal ligation of the common bile duct, a polythene catheter (Portex Limited, Hythe, England), with an inner diameter of 0.28 mm, was directly inserted into the exposed gallbladder. The catheter was fixed to the gallbladder with an additional ligation. [^{3}H]-digoxin was injected i.v. at a dose of 0.2 mg kg^{-1} and bile was collected for 90 min. After these 90 min blood was collected from the axillary blood vessels and tissues and intestinal contents were collected and prepared for scintillation counting as described above. Three female mice were analysed in each group.

Determination of digoxin and its pharmacologically active metabolites

An automatic Fluorescence Polarisation Immunoassay (TDxFLx, Abbott Laboratories, North Chicago, IL, U.S.A.) was used. As tested by the manufacturer (TDxFLx assays manual), the TDxFLx assay can distinguish the pharmacologically active compounds (digoxin, bis-digitoxoside, mono-digitoxoside, digoxigenin) from inactive metabolites (such as dihydrodigoxigenin and dihydrodigoxin). The assay cannot distinguish between digoxin itself and its pharmacologically active metabolites (see above), as the latter compounds are highly cross-reactive (140% for bis-digitoxoside, 180% for mono-digitoxoside, 97% for digoxigenin) relative to the parent drug. The actual concentrations of total pharmacologically active compounds in our study may therefore be somewhat lower than indicated, since bis-digitoxoside and mono-digitoxoside are the two major metabolites of digoxin in rodents, as was shown in previous studies (von Bergmann *et al.*, 1972).

Sample preparation for TDxFLx measurements

Plasma, urine and bile were diluted in human plasma to obtain concentrations below 5 ng ml^{-1}. The assay was calibrated for concentrations from 0.5–5 ng ml^{-1}, the detection limit was 0.2 ng ml^{-1}. For brain and faeces homogenates a lipid extraction with ethanol/water 1:1 was carried out: 200 μl homogenate and 400 μl ethanol/water were mixed for 10 min and centrifuged; 200 μl of the supernatant was evaporated in a vacuum centrifuge. After redissolving in 200 μl human plasma, the sample was ready for measurement. The recovery of radioactivity in the supernatant with this procedure was between 80–85% compared to the homogenate.

Drugs

[^{3}H(G)]-digoxin (16.0 Ci mmol^{-1}) was obtained from DuPont NEN, Boston, MA, U.S.A. Hypnorm was from Janssen Pharmaceuticals B.V., Tilburg. Dormicum was from Roche Nederland B.V., Mijdrecht. Digoxin was from Sigma Chemical Co., St. Louis, MO, U.S.A.

Statistical analysis

Where appropriate, Student's independent t test was used to test the significance of a difference in the means of two sets of data. $P<0.05$ was considered statistically significant.

Results

Drug excretion

After i.v. administration the route of excretion of [^{3}H]-digoxin was remarkably different in *mdr1a* (−/−) and wild-type mice,

as illustrated in Table 1. Whereas more than half of [^{3}H]-digoxin was excreted with the faeces in wild-type mice, these levels dropped markedly in *mdr1a* (−/−) mice, while urinary elimination was increasing more than 2 fold. Although a faecal excretion was more prominent in both mouse strains after oral administration of [^{3}H]-digoxin, we again observed a marked shift towards urinary excretion in *mdr1a* (−/−) mice (Table 1). Most of the radioactivity was excreted during the first 24 h, although *mdr1a* (−/−) mice showed some delay in this respect (Table 2). The fraction of pharmacologically active compounds in urine collected over 24 h after drug administration was more than 60% of the radioactivity recovered and comparable to that in plasma, whereas in faeces this fraction was lower (~35%) (Table 3).

Rodents are relatively resistant to the toxic effects of digoxin (Hoffman & Lendle, 1953). In spite of this, the *mdr1a* (−/−) mice showed some signs of toxicity at a dose of 0.2 mg [^{3}H]-digoxin per kg. Besides moderate passivity, lack of feeding and decreased production of faeces during the first 24 h were observed. After i.v. injection of [^{3}H]-digoxin, bulk excretion of faeces was decreased by 60–90%. After oral administration there was a higher interindividual variation in faeces production in *mdr1a* (−/−) mice (from normal production to 80% decrease). Toxicity was transient and all mice completely recovered within 24 h after drug administration. To exclude the possibility that the altered excretion route observed in the *mdr1a* (−/−) mice resulted mainly from the toxic side effects of digoxin, we tested excretion at an i.v. dose of 0.05 mg kg^{-1}. At this dose level, no significant toxicity was detectable in *mdr1a* (−/−) mice, and feeding and faeces production were comparable to wild-type mice. Nevertheless, Table 1 shows that at 0.05 mg kg^{-1}, the patterns of excretion of [^{3}H]-digoxin were very similar to those observed at 0.2 mg kg^{-1}, indicating that the shift from faecal to urinary excretion in *mdr1a* (−/−) mice was not primarily caused by toxic side effects.

As the enterohepatic circulation of cardiac glycosides is substantial in rodents (Lauterbach, 1964; Abshagen *et al.*, 1972), both decreased biliary excretion and increased intestinal re-uptake of [^{3}H]-digoxin in *mdr1a* (−/−) mice could contribute to the drop in faecal excretion. To discriminate between these two mechanisms, we analyzed [^{3}H]-digoxin excretion in anaesthetized mice with a ligated common bile duct, thus interrupting the biliary excretion into the gut lumen.

The most striking result of these experiments was that more than 16% of the administered [^{3}H]-digoxin was directly excreted into the intestinal lumen of wild-type mice within 90 min, in contrast to only about 2% in *mdr1a* (−/−) mice (Table 4). The majority of the excreted radioactivity in wild-type mice was found in the small intestine and caecum. The biliary excretion in both wild-type and *mdr1a* (−/−) mice was substantial, and not significantly different between both strains ($P=0.22$) (Table 4). The immunoassay for both the collected

Table 1 Urinary and faecal excretion of radioactivity over 72 h after i.v. or oral administration of [^{3}H]-digoxin

		Wild-type	*mdr1a (−/−)*
		(% of administered dose)	
[^{3}H]-digoxin 0.2 mg kg^{-1}			
Urine	i.v.	33.2±6.8	73.1±1.2**
Faeces	i.v.	58.1±7.7	14.8±1.3**
Urine	oral	13.7±1.9	46.4±6.2**
Faeces	oral	78.2±0.8	40.6±4.9**
[^{3}H]-digoxin 0.05 mg kg^{-1}			
Urine	i.v.	26.1±4.7	68.4±3.8**
Faeces	i.v.	50.4±3.5	16.5±2.2**

[^{3}H]-digoxin was administered i.v. or orally at a dose of 0.2 mg kg^{-1} or i.v. at a dose of 0.05 mg kg^{-1}. Levels of radioactivity are expressed as means±s.e.mean ($n=3$); **$P<0.01$ for each pair of data

Table 2 Urinary and faecal excretion of radioactivity over 24 h after i.v. or oral administration of [^{3}H]-digoxin (0.2 mg kg^{-1})

		Wild-type	*mdr1a (−/−)*
Urine (%)	i.v.	30.9±5.4	52.7±5.3*
Faeces (%)	i.v.	46.9±6.4	5.2±0.3**
Plasma (ng ml^{-1})	i.v.	3±0.4	26±2**
Urine (%)	oral	18.9±2.9	60.4±2.8**
Faeces (%)	oral	75.8±2.3	21.9±4.0**
Plasma (ng ml^{-1})	oral	2±0.4	18±6

Excretion of radioactivity as percentage of administered dose±s.e.mean ($n=3$); *$P<0.05$, **$P<0.01$ for each pair of data

Table 3 Urinary and faecal excretion of digoxin and pharmacologically active metabolites over 24 h

		Wild-type	*mdr1a (−/−)*
Urine (%)	i.v.	23.9±4.6	45.0±4.7*
Faeces (%)	i.v.	15.4±3.9	2.4±0.6**
Plasma (ng ml^{-1})	i.v.	2±0.4	19±1**
Urine (%)	oral	12.8±2.6	48.7±3.9**
Faeces (%)	oral	27.5±5.1	9.0±3.0*
Plasma (ng ml^{-1})	oral	1±0.4	13±5

[^{3}H]-digoxin was administered i.v. or orally at a dose of 0.2 mg kg^{-1}. Levels for urine and faeces are expressed as percentage of administered dose±s.e.mean ($n=3$); *$P<0.05$, **$P<0.01$ for each pair of data

Table 4 Tissue levels and intestinal and biliary excretion of [^{3}H]-digoxin-derived radioactivity in mice with a cannulated gallbladder

	Wild-type	*mdr1a (−/−)*	*Ratio* (*mdr1a (−/−)*: wild-type)
Brain (ng g^{-1})	8±3	123±29**	15
Liver (ng g^{-1})	338±68	580±48*	1.7
Plasma (ng ml^{-1})	125±10	216±14**	1.7
(% of administered dose)			
Contents small intestine	8.0±1.0	1.7±0.3**	0.21
Contents caecum	6.2±0.7	0.3±0.1**	0.05
Contents colon	2.2±1.1	0.2±0.1	0.09
Total intestine	16.4±2.6	2.2±0.4**	0.13
Biliary excretion	24.0±4.8	15.8±2.9	0.66

After cannulation of the gallbladder and ligation of the common bile duct, mice received an i.v. injection of [^{3}H]-digoxin (0.2 mg kg^{-1}). Bile was collected over 90 min after [^{3}H]-digoxin administration. Levels are expressed as means±s.e.mean ($n=3$); *$P<0.05$, **$P<0.01$

bile and intestinal contents showed that at least 80–90% of the excreted radioactivity represented digoxin or closely related, pharmacologically active metabolites (data not shown).

Tissue and brain distribution

Table 5 shows a tissue distribution panel for [^{3}H]-digoxin in *mdr1a* (−/−) mice and wild-type mice 8 h after i.v. administration of 0.2 mg kg^{-1}. The relative [^{3}H]-digoxin levels in most tissues reflected the roughly 4 fold higher plasma level in *mdr1a* (−/−) mice, brain being the exception with a highly increased level of [^{3}H]-digoxin. A comparison of the plasma and brain concentrations in the course of time in *mdr1a* (−/−) and wild-type mice is shown in Figure 1a and b (after i.v. bolus injection) and Figure 2a and b (after oral bolus administration). In the brain of wild-type mice [^{3}H]-digoxin levels remained very low, whereas in *mdr1a* (−/−) mice these levels gradually increased over a period of 3 days, resulting in a ~200 fold difference from wild-type mice after 72 h. At the time of maximum drug concentrations in brain (~72 h after both i.v. and oral administration), plasma levels of [^{3}H]-digoxin were already close to the detection limit. The immunoassay showed that at 4, 72 and 120 h after i.v. administration, levels of digoxin and pharmacologically active metabolites represented respectively 87.3±6.5%, 60.6±3.1%, and 92.0±0.9% of the total radioactivity (±s.e.mean) detected in the brain of *mdr1a* (−/−) mice. Similarly, 72 h after oral administration this percentage was 59.5±15.8%.

Table 5 Tissue concentrations of radioactivity in mdr1a (−/−) and wild-type mice 8 h after i.v. administration of [^{3}H]-digoxin (0.2 mg kg^{-1})

Tissue	(ng g^{-1} tissue or ng ml^{-1} plasma) Wild-type	*mdr1a (−/−)*	*Ratio mdr1a (−/−): wild-type*
Brain	3.5±0.8	231±8**	66
Fat (neck)	17±7	57±4**	3.5
Muscle	14±5	71±2**	5.0
Liver	44±16	211±33*	4.8
Gallbladder	1166±218	8890±804**	7.6
Kidney	14±4	43±4**	3.0
Lung	10±3	36±0.7**	3.6
Spleen	6.9±2	22±4*	3.2
Heart	12±5	49±6**	4.3
Thymus	10±3	19±4	2.0
Ovaries	28±7	53±8	1.9
Eyes	22±4	84±10**	3.9
Plasma	16±6	67±12*	4.2

Results are expressed as means±s.e.mean ($n=3$); *$P<0.05$, **$P<0.01$

Discussion

We show here that intestinal mdr1a P-gp can excrete 16% of i.v. administered [^{3}H]-digoxin within 90 min into the gut lumen of wild-type mice, demonstrating a substantial drug elimination from the systemic circulation. It is further noteworthy that the total amount of faecally excreted radioactivity in *mdr1a* (−/−) mice over 72 h after i.v. drug administration (14.8%, Table 1) is not more than the hepatobiliary excretion over 90 min (15.8%, Table 4). This suggests that at least part of the radioactivity excreted in the bile is reabsorbed in the intestine of *mdr1a* (−/−) mice. Based on the demonstrated vectorial transport activity of mdr1a P-gp in wild-type intestine, it is very likely that this reabsorption process occurs more efficiently in *mdr1a* (−/−) than in wild-type intestine. Thus, in all likelihood, intestinal P-gp activity favours faecal excretion of a

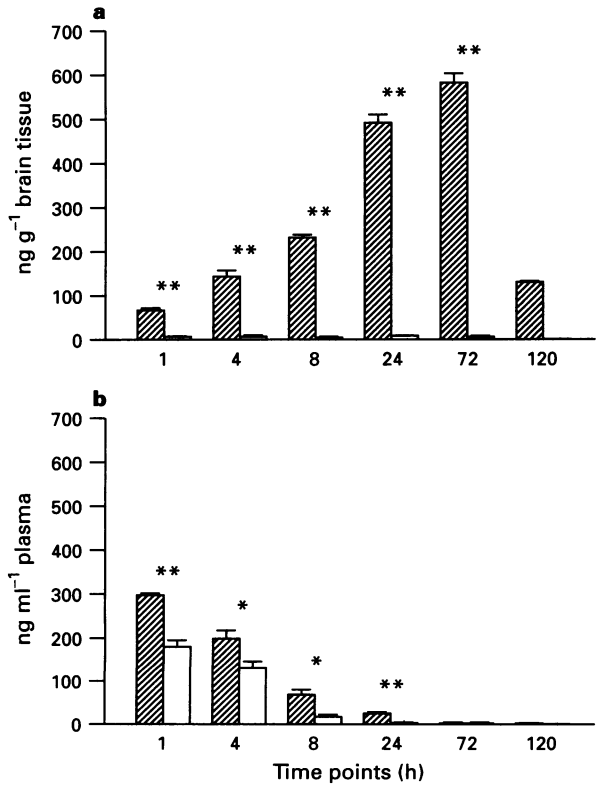

Figure 1 Brain (a) and plasma (b) levels of radioactivity in *mdr1a* (−/−) mice (hatched columns) and wild-type mice (open columns) after i.v. administration of [^{3}H]-digoxin (0.2 mg kg^{-1}). Mean levels with s.e.mean are depicted (n=3). Brain and plasma levels at 120 h were not determined for wild-type mice. *P<0.05, **P<0.01 for each pair of columns.

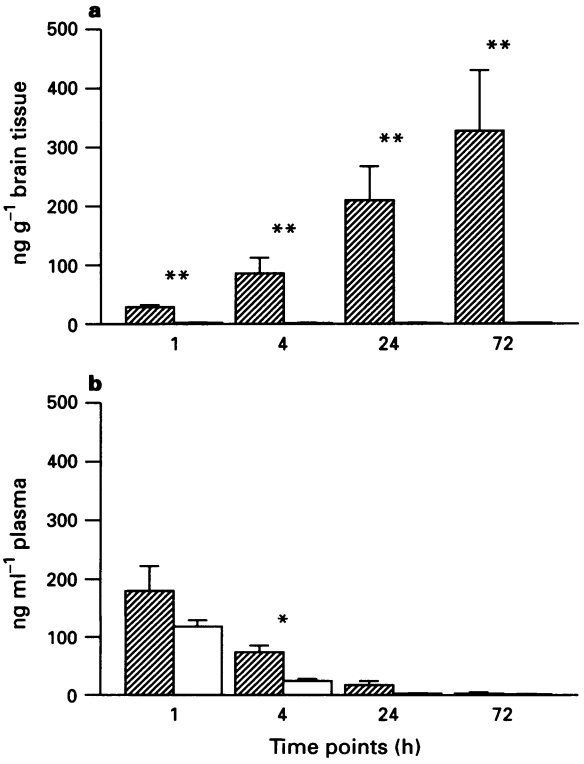

Figure 2 Brain (a) and plasma (b) levels of radioactivity in *mdr1a* (−/−) mice (hatched columns) and wild-type mice (open columns) after oral administration of [^{3}H]-digoxin (0.2 mg kg^{-1}). Mean levels with s.e.mean are depicted (n=3). *P<0.05, **P<0.01 for each pair of columns.

substrate drug both by directly excreting the drug into the intestinal lumen, and by limiting the rate of re-uptake of the drug after it entered the intestine by biliary excretion. The direct excretion of compounds via the intestinal epithelium by P-gp explains previous findings suggesting that this route of drug elimination is significant for several cardiac glyosides in various animal species and in human subjects (Lauterbach, 1969; Selden *et al.*, 1974). In addition, Su & Huang (1996) recently demonstrated that in rats, digoxin excretion via the intestinal epithelium can be inhibited substantially by co-administered quinidine, an effective reversal agent for P-gp (Ford & Hait, 1990). Our data support the hypothesis that these effects are due primarily to inhibition of P-gp, and not of other transporters.

Previous studies in human subjects and rodents have shown that biliary excretion is important for several cardiac glycosides and their metabolites (Marcus *et al.*, 1964; Rietbrock *et al.*, 1972). Initially we had therefore expected that the absence of mdr1a P-gp normally present in the biliary canalicular membrane of the hepatocytes (Thiebaut *et al.*, 1987) would be the main factor responsible for the altered digoxin excretion in *mdr1a* (−/−) mice. However, biliary excretion was not decreased in *mdr1a* (−/−) mice. Other transporters must therefore be involved in biliary digoxin excretion as well. The data illustrate that the lower faecal elimination of [^{3}H]-digoxin in *mdr1a* (−/−) mice is due to a drop in drug excretion via the intestinal epithelium rather than due to a decrease in biliary excretion of the drug.

In spite of the absence of mdr1a P-gp from the kidney of *mdr1a* (−/−) mice, the cumulative urinary excretion of digoxin in these mice was increased compared to wild-type mice. This could be due to the activity of other transporters (e.g. mdr1b P-gp, for which the RNA level was increased in the kidney of *mdr1a* (−/−) mice, Schinkel *et al.*, 1994). However, in various animal species and human subjects as well, glomerular filtration is also involved in the renal handling of digoxin (Steiness, 1974; Broen-Christensen *et al.*, 1977). This means that even in the total absence of active drug transporters, there could still be a substantial renal excretion capacity for digoxin. The shutting off of the intestinal route of digoxin elimination in *mdr1a* (−/−) mice shifts the balance of excretion to the still open route of urinary elimination. Together, our data demonstrate the importance of the intestinal mdr1a P-gp activity for the overall pattern of excretion of [^{3}H]-digoxin in mice.

Compared to human subjects, rodents are highly resistant to the pharmacodynamic effects of cardiac glycosides. Only at a dose close to the LD_{50} of α-acetyl-digoxin in rats (4.8 mg kg^{-1}) was paralysis of the intestine observed in *in situ* experiments on isolated intestinal loops (Hoffman & Lendle, 1953). Such peripheral toxicity of digoxin could cause constipation explaining the decrease in faeces production that we found in *mdr1a* (−/−) mice.

However, we cannot exclude the possibility that other toxic effects, such as direct CNS-toxicity, were responsible for the observed side-effects of digoxin treatment, as we found a remarkable accumulation of [^{3}H]-digoxin in the brain of *mdr1a* (−/−) mice. Compared to plasma and other organs (not shown), where levels of [^{3}H]-digoxin were already decreasing from 1 h to 4 h after drug administration, brains of *mdr1a* (−/−) mice continued to accumulate [^{3}H]-digoxin. Only after 3 days, when plasma levels fell below ~2 ng ml^{-1}, did the drug levels in brain start dropping (Figure 1a, b). This high and long lasting accumulation of radioactivity was not due to degradation products as at least 60% of radioactive material was composed of digoxin and pharmacologically active metabolites at various time points. We attribute the accumulation to the high digoxin binding capacity and affinity of the brain compared to other organs. It was previously shown in animals with an intact blood-brain barrier that digoxin, although at very low levels, accumulated in brain after repeated drug administration, and was only slowly released. The effect was even stronger for methyl-digoxin (Kuhlmann *et al.*, 1979). As proposed by Flasch & Heinz (1976), the ability of compounds to bind to brain tissue will probably decrease with decreasing hydrophobicity. It should be noted that not all drugs affected by blood-brain barrier P-gp continue to accumulate in the brain of *mdr1a* (−/−) mice, as was demonstrated for vinblastine (Schinkel *et al.*, 1994; Van Asperen *et al.*, 1996) and cyclosporin A (Schinkel *et al.*, 1995). Nevertheless, both increased brain entry and increased brain retention should be taken into account when P-gp substrates are administered in combination with an effective reversal agent (e.g. SDZ PSC 833). For instance, in animal experiments, the administration of various cytostatic drugs after the pharmacological opening of the blood-brain barrier led to severe functional and histological neurotoxicity (Neuwelt *et al.*, 1983). Also in patients, increased local toxicity of chemotherapeutic agents has been observed by raising drug concentrations in the brain (Kaplan & Wiernik, 1982; Siegers, 1990).

The fact that the Fluorescence Polarization Immunoassay for digoxin cross-reacts with the pharmacologically active metabolites, bis-digitoxoside (140%), mono-digitoxoside (180%) and digoxigenin (97%) (see Methods), limits the quantitative precision of the data obtained using this assay. Since previous studies in rodents have shown that at least over a period of 12 h after drug administration, only about 20% of digoxin appeared as bis- and mono-digitoxoside in urine and bile, whereas 60–70% remained unchanged (Abshagen *et al.*, 1972; von Bergmann *et al.*, 1972), this cross-reactivity would probably affect only slightly our measurements of pharmacologically active compounds. Moreover, even in the theoretical case of complete metabolism to mono-digitoxoside taking place exclusively in *mdr1a* (−/−) and not in wild-type mice, this would still hardly influence our conclusions, in view of the profound differences we found between both mouse strains for the most relevant pharmacokinetic parameters (i.e., direct intestinal drug excretion and brain distribution of the drug).

In summary, we have shown that intestinal P-gp in mice contributes to a substantial excretion of [^{3}H]-digoxin via the gut epithelium. This is not only of importance for drug elimination from the systemic circulation, but very likely also for a decreased intestinal re-uptake of digoxin after biliary excretion (enterohepatic circulation). The prolonged accumulation of [^{3}H]-digoxin in brains of *mdr1a* (−/−) mice extends our previous findings (Schinkel *et al.*, 1994; 1995), and it further emphasizes the pharmacological importance of P-gp in the blood-brain barrier. It should make physicians aware of a possibly increased risk for CNS-toxicity in patients exposed to co-administration of effective reversal agents with digoxin or other drugs transported by P-gp.

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References

ABSHAGEN, U., VON BERGMANN, K. & RIETBROCK, N. (1972). Evaluation of the enterohepatic circulation after ^{3}Hdigoxin administration in the rat. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **275**, 1–10.

BALDINI, N., SCOTLANDI, K., BARBANTI-BRÓDANO, G., MANARA, M.C., MAURICI, D., BACCI, G., BERTONI, F., PICCI, P., SOTTILI, S., CAMPANACCI, M. & SERRA, M. (1995). Expression of P-glycoprotein in high-grade osteosarcomas in relation to clinical outcome. *N. Engl. J. Med.*, **333**, 1380–1385.

BELLAMY, W.T., DALTON, W.S. & DORR, R.T. (1990). The clinical relevance of multidrug resistance. *Cancer Invest.*, **8**, 547–562.

BORST, P. & SCHINKEL, A.H. (1996). Mice with disrupted P-glycoprotein genes. In *Multidrug Resistance in Cancer Cells*. ed. Gupta, S. & Tsuruo, T. Chichester, Sussex: John Wiley & Sons, Ltd (in press).

BOURHIS, J., BÉNARD, J., HARTMANN, O., BOCCON-GIBOD, L., LEMERLE, J. & RIOU, G. (1989). Correlation of MDR1 gene expression with chemotherapy in neuroblastoma. *J. Natl. Cancer Inst.*, **81**, 1401–1405.

BROEN-CHRISTENSEN, C., JANSEN, J.A. & STEINESS, E. (1977). Renal excretion of digoxin in rats and rabbits. *Acta Pharmacol. Toxicol.*, **41** [Suppl. IV], 43 (abstr.)

CORDON-CARDO, C., O'BRIEN, J.P., CASALS, D., RITTMAN-GRAUER, L., BIEDLER, J.L., MELAMED, M.R. & BERTINO, J.R. (1989). Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 695–698.

DALTON, W.S., CROWLEY, J.J., SALMON, S., GROGAN, T.M., ROGERS LAUFMANN, L., WEISS, G.R. & BONNET, J.D. (1995). A phase III randomized study of oral verapamil as a chemosensitizer to reverse drug resistance in patients with refractory myeloma: a Southwest Oncology Group study. *Cancer*, **75**, 815–820.

DE LANNOY, I.A.M., KOREN, G., KLEIN, J., CHARUK, J. & SILVERMAN, M. (1992). Cyclosporin and quinidine inhibition of renal digoxin excretion: evidence for luminal secretion of digoxin. *Am. J. Physiol.*, **263**, F613–F622.

DE LANNOY, I.A.M. & SILVERMAN, M. (1992). The MDR1 gene product, P-glycoprotein, mediates the transport of the cardiac glycoside, digoxin. *Biochem. Biophys. Res. Commun.*, **189**, 551–557.

FLASCH, H. & HEINZ, N. (1976). Konzentration von Herzglykosiden im Myokard und im Gehirn. *Arzneim. Forsch. (Drug Res.)*, **26**, 1213–1216.

FORD, J.M. & HAIT, W.N. (1990). Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol. Rev.*, **42**, No. 3, 155–199.

GOTTESMAN, M.M. & PASTAN, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.*, **62**, 385–427.

HAGER, W.D., FENSTER, P., MAYERSOHN, M., PERRIER, D., GRAVES, P., MARCUS, F.I. & GOLDMAN, S. (1979). Digoxin-quinidine interaction. *N. Engl. J. Med.*, **300**, 1238–1241.

HOFFMANN, G. & LENDLE, L. (1953). Nachweis extrakardialer Digitaliswirkungen an der Ratte. *Naunyn Schmiedeberg's Arch. Exp. Path. Pharmakol.*, **217**, 184–193.

HORI, R., OKAMURA, N., AIBA, T. & TANIGAWARA, Y. (1993). Role of P-glycoprotein in renal tubular secretion of digoxin in the isolated perfused rat kidney. *J. Pharmacol. Exp. Ther.*, **266**, 1620–1625.

ITO, S., WOODLAND, C., HARPER, P.A. & KOREN, G. (1993). The mechanism of the verapamil-digoxin interaction in renal tubular cells (LLC-PK1). *Life Sci.*, **53**, PL399–PL403.

JULIANO, R.L. & LING, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta*, **455**, 152–162.

KAPLAN, R.S. & WIERNIK, P.H. (1982). Neurotoxicity of antineoplastic drugs. *Semin. Oncol.*, **9**, 103–130.

KUHLMANN, J., RIETBROCK, N. & SCHNIEDERS, B. (1979). Tissue distribution and elimination of digoxin and methyldigoxin after single and multiple doses in dogs. *J. Cardiovasc. Pharmacol.*, **1**, 219–234.

LAUTERBACH, F. (1964). Enterale Resorption, biliäre Ausscheidung und entero-hepatischer Kreislauf von Herzglykosiden bei der Ratte. *Naunyn-Schmiedeberg's Arch. Exp. Path. Pharmakol.*, **247**, 391–411.

LAUTERBACH, F. (1969). Die enterale Sekretion kardiotoner Steroide–Untersuchungen zum Mechanismus des Resorptionsvorganges. *Naunyn-Schmiedeberg's Arch. Pharmak.*, **263**, 267–268.

MARCUS, F.I., KAPADIA, G.J. & KAPADIA, G.G. (1964). The metabolism of digoxin in normal subjects. *J. Pharmacol. Exp. Ther.*, **145**, 203–209.

MARIE, J.P., ZITTOUN, R. & SIKIC, B..I. (1991). Multidrug resistance (MDR1) gene expression in adult acute leukemias: Correlations with treatment outcome and in vitro drug sensitivity. *Blood*, **78**, 586–592.

MOYSEY, J.O., JAGGAROA, N.S.V., GRUNDY, E.N. & CHAMBERLAIN, D.A. (1981). Amiodarone increases plasma digoxin concentrations. *Br. Med. J.*, **282**, 272.

NEUWELT, E.A., GLASSBERG, M., FRENKEL, E. & BARNETT, P. (1983). Neurotoxicity of chemotherapeutic agents after blood-brain barrier modification: Neuropathological studies. *Ann. Neurol.*, **14**, 316–324.

OKAMURA, N., HIRAI, M., TANIGAWARA, Y., TANAKA, K., YASUHARA, M., UEDA, K., KOMANO, T. & HORI, R. (1993). Digoxin-cyclosporin A interaction: Modulation of the multidrug transporter P-glycoprotein in the kidney. *J. Pharmacol. Exp. Ther.*, **266**, 1614–1619.

PINEDO, H.M. & GIACCONE, G. (1995). P-glycoprotein–A marker of cancer cell behaviour. *N. Engl. J. Med.*, **333**, 1417–1419.

RIETBROCK, N., ABSHAGEN, U., VON BERGMANN, K. & KEWITZ, H. (1972). Pharmacokinetics of digoxin and its 4'''-acetyl- and methylderivates in the rat. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **274**, 171–181.

SCHINKEL, A.H., SMIT, J.J.M., VAN TELLINGEN, O., BEIJNEN, J.H., WAGENAAR, E., VAN DEEMTER, L., MOL, C.A.A.M., VAN DER VALK, M.A., ROBANUS-MAANDAG, E.C., TE RIELE, H.P.J., BERNS, A.J.M. & BORST, P. (1994). Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood brain barrier and to increased sensitivity to drugs. *Cell*, **77**, 491–502.

SCHINKEL, A.H., WAGENAAR, E., VAN DEEMTER, L., MOL, C.A.A.M. & BORST, P. (1995). mdr1a P-glycoprotein in mice affects tissue distribution and pharmacology of cyclosporin A, digoxin and dexamethasone. *J. Clin. Invest.*, **96**, 1698–1705.

SCHWARTZ, J.B., KEEFE, D., KATES, R.E., KIRSTEN, E. & HARRISON, D.C. (1980). Acute and chronic pharmacodynamic interaction of verapamil and digoxin and atrial fibrillation. *Circulation*, **65**, 1163–1170.

SELDEN, R., MARGOLIES, M.N. & SMITH, T.W. (1974). Renal and gastrointestinal excretion of ouabain in dog and man. *J. Pharmacol. Exp. Ther.*, **188**, 615–623.

SIEGERS, H.P. (1990). Chemotherapy for brain metastases: recent developments and clinical considerations. *Cancer Treat. Rev.*, **17**, 63–76.

SIKIC, B.I. (1995). Clinical modulation of multidrug resistance. *AACR-meeting 'Novel Strategies against Resistant Cancers'*, Florida 17–21 Nov. 1995. (abstr.)

SIKIC, B.I., FISHER, G.A., LUM, B.L., BROPHY, N.A., YAHANDA, A.M., ADLER, K.M. & HALSEY, J. (1994). Clinical reversal of multidrug resistance. In *Anticancer Drug Resistance: Advances in Molecular and Clinical Research*. ed. Goldstein, L.J. & Ozols, R.F. pp. 149–165. Boston/Dordrecht/London: Kluwer Academic Publishers.

STEINESS, E. (1974). Renal tubular secretion of digoxin. *Circulation*, **50**, 103–107.

SU, S.F. & HUANG, J.D. (1996). Inhibition of the intestinal digoxin absorption and exsorption by quinidine. *Drug Metab. Dispos.*, **24**, 142–147.

TANIGAWARA, Y., OKAMURA, N., HIRAI, M., YASUHARA, M., UEDA, K., KIOKA, N., KOMANO, T. & HORI, R. (1992). Transport of digoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK). *J. Pharmacol. Exp. Ther.*, **263**, 840–845.

THIEBAUT, F., TSURUO, T., HAMADA, H., GOTTESMAN, M.M., PASTAN, I. & WILLINGHAM, M.C. (1987). Cellular localization of the multidrug resistance gene product in normal human tissues. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7735–7738.

TSURUO, T., IIDA, H., TSUKAGOSHI, S. & SAKURAI, Y. (1981). Overcoming vincristine resistance in P388 leukemia *in vivo* and *in vitro* trough enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res.*, **41,** 1967–1972.

TWENTYMAN, P.R. & BLEEHEN, N.M. (1991). Resistance modification by PSC-833, a novel non-immunosuppressive cyclosporin A. *Eur. J. Cancer*, **27,** 1639–1642.

VAN ASPEREN, J., SCHINKEL, A.H., BEIJNEN, J.H., NOOIJEN, W.J., BORST, P. & VAN TELLINGEN, O. (1996). Altered pharmacokinetics of vinblastine in mdr1a P-glycoprotein deficient mice. *J. Natl. Cancer Inst.*, **88,** 994–999.

VON BERGMANN, K., ABSHAGEN, U. & RIETBROCK, N. (1972). Quantitative analysis of digoxin, 4-acetyldigoxin, and 4-methyldigoxin and their metabolites in bile and urine of rats. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **273,** 154–167.

WILSON, W.H., BATES, S.E., FOJO, A., BRYANT, G., ZHAN, Z., REGIS, J., WITTES, R.E., JAFFE, E.S., STEINBERG, S.M., HERDT, J. & CHABNER, B.A. (1995). Controlled trial of dexverapamil, a modulator of multidrug resistance, in lymphomas refractory to EPOCH chemotherapy. *J. Clin. Oncol.*, **13,** 1995–2004.

WISHART, G.C., BISSET, D., PAUL, J., JODRELL, D., HARNETT, A., HABESHAW, T., KERR, D.J., MACHAM, M.A., SOUKOP, M., LEONARD, R.C.F., KNEPIL, J. & KAYE, S.B. (1994). Quinidine as a resistance modulator of epirubicin in advanced breast cancer: mature results of a placebo-controlled randomized trial. *J. Clin. Oncol.*, **12,** 1771–77.

YUEN, A.R. & SIKIC, B.I. (1994). Multidrug resistance in lymphomas. *J. Clin. Oncol.*, **12,** 2453–2459.

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