THE ROLE OF MDR1A P-GLYCOPEPTIDE IN THE BILIARY AND INTESTINAL SECRETION OF DOXORUBICIN AND VINBLASTINE IN MICE

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ABSTRACT:

Drug-transporting P-glycoproteins are abundantly present in the liver and the intestinal wall. We have now investigated their role in the biliary and intestinal secretion of the anticancer drugs doxorubicin (unlabeled: 5 mg/kg) and vinblastine (3H-labeled: 1 mg/kg) i.v. administered to wild-type and mdr1a P-glycoprotein knockout (mdr1a−/−) mice. At 90 min after drug administration, levels of unchanged drug and metabolites in plasma, intestinal contents, and bile were determined by high-performance liquid chromatography and radioactivity by liquid scintillation counting. The bile of both wild-type and mdr1a−/− mice contained only minor amounts of unchanged vinblastine, whereas the total biliary secretion of unchanged 3H-labeled breakdown products was about 25 to 30% of the dose. The direct secretion of unchanged vinblastine through the gut wall was 6.7 and 3.3% of the dose in wild-type and mdr1a−/− mice, respectively. The biliary secretion of unchanged doxorubicin decreased from 13.3% of the dose to only 2.4% in the absence of mdr1a P-glycoprotein. Approximately 10% of the dose was secreted as unchanged doxorubicin into the intestinal contents of both types of mice. Thus, the absence of mdr1a P-glycoprotein affects the fate of vinblastine chiefly by diminishing secretion into the lumens of the small intestine, whereas it affects the fate of doxorubicin chiefly by diminishing secretion of parent drug into bile.

The drug-transporting P-glycoprotein encoded by the human MDR1 gene is a large membrane-associate protein that actively transports a broad range of substrates out of the cell, including many anticancer drugs, e.g., Vinca alkaloids, taxanes, anthracyclines, and epipodophyllotoxins. It was initially discovered as a marker of multidrug resistance in mammalian tumor cells (Juliano and Ling, 1976). Subsequent studies demonstrated that this protein is also present in many normal tissues and that it is highly conserved in size, immunologically cross-reactivity, and amino acid sequence across species, which is indicative of a fundamentally important function (reviewed in Endicott and Ling, 1989).

The tissue-specific localization of MDR1 P-glycoprotein formed the basis for the initial ideas about a protective function (Thiebaut et al., 1987; Cordon-Cardo et al., 1989). MDR1 P-glycoprotein at the brush border (luminal side) of enterocytes, lining the gastrointestinal tract and mediating transport toward the gut lumen, is thought to play a role in the protection of the organism against orally ingested toxins. Its presence in the blood-brain and the blood-testis barrier and in the placenta may limit the entry of toxins into these compartments, whereas MDR1 P-glycoprotein in the proximal tubules of the kidney and in the bile canalicus of hepatocytes might contribute to the elimination of toxins from the body.

The existence of additional physiological functions of MDR1 P-glycoprotein is still debated (Borst et al., 1998). To study the physiological and pharmacological role of the drug-transporting P-glycoproteins, mice with disrupted P-glycoprotein genes have been generated in our institute (Schinkel et al., 1994, 1997). In contrast to humans, mice have two genes (mdr1a and mdr1b) that code for drug-transporting P-glycoproteins, and together these probably fulfill the same role as MDR1 P-glycoprotein in humans. The mdr1a gene is predominantly expressed in the intestinal epithelium and in the capillaries of the brain and the testis, whereas mdr1b P-glycoprotein is mainly present in the adrenal gland and ovaries. Tissues with significant levels of both proteins include liver, kidney, lung, heart, and spleen (Croz et al., 1989). Mouse mdr2 and its human homolog MDR3 P-glycoprotein are not involved in drug transport. Instead they serve as the phosphatidylcholine translocator in the biliary canalicular membrane, and the absence of the murine mdr2 P-glycoprotein results in chronic progressive liver disease (Smit et al., 1993; Smith et al., 1998). Mice lacking the drug-transporting P-glycoproteins are viable and fertile and have a normal life span, suggesting that the drug-transporting P-glycoproteins do not have any physiological function. Although it has been reported that drug-transporting P-glycoproteins can translocate a variety of short-chain phospholipids from the inner to the outer leaflet of the plasma membrane, they are probably not capable of translocating long-chain phosphatidylcholine (van Helvoort et al., 1996). The recent pharmacokinetic analysis of P-glycoprotein substrate drugs in mice with disrupted P-glycoprotein genes has established the protective role of P-glycoprotein against toxic xenobiotics, because these mice are much more sensitive to toxic substrates. P-glycoprotein protection takes place at the level of individual organs, with markedly increased drug accumulation in the brain as the most clear-cut example, as well as at the level of the whole body by diminished clearance or absorption (Schinkel et al., 1994, 1995, 1996, 1997).
Mice with a homozygous disruption of the mdr1a gene (mdr1a<sup>−/−</sup>) mice) were previously used to investigate the role of P-glycoprotein in the pharmacokinetics of two important anticancer agents that are substrates for this drug-transporting protein, vinblastine (van Asperen et al., 1996) and doxorubicin (van Asperen et al., 1999). The fecal excretion of unchanged vinblastine was significantly lower in mdr1a<sup>−/−</sup> mice compared with wild-type mice, whereas the fecal excretion of doxorubicin was similar in both types of mice. However, as both biliary secretion and direct secretion via the gut wall can contribute to fecal elimination, the present experiments were conducted to further unravel the role of mdr1a P-glycoprotein in each of these secretion pathways for vinblastine and doxorubicin. For that purpose, the dose levels of the drugs, and the strain and gender of the animals were similar to the lowest dose levels used in the previous studies. Drug-related side effects were absent and saturation of metabolic enzymes and transporters did not seem to occur at these relatively low doses. By analogy with a previous study with paclitaxel (Sparreboom et al., 1997), wild-type and mdr1a<sup>−/−</sup> mice with a cannulated gallbladder were used to discriminate between these two secretion pathways.

Materials and Methods

Animals. The experiments with vinblastine and doxorubicin were performed with male and female mice, respectively. FVB wild-type and mdr1a<sup>−/−</sup> mice between 10 and 15 weeks of age were used in both experiments. They were housed and handled according to institutional guidelines. Food (Hope Farms B.V., Woerden, the Netherlands) and acidified water were given ad libitum.

Drugs and Chemicals. Doxorubicin·HCl (Adriblastina; Pharmacia Netherlands, Woerden, the Netherlands) was dissolved at 2 mg of doxorubicin·HCl/ml saline (NPBI B.V., Emmer-Compascuum, the Netherlands). Doxorubicinol, 7-deoxydoxorubicinolone, and 7-deoxydoxorubicinone were provided by Pharmacia-Farmitalia-Carlo Erba. Vinblastine sulfate was obtained from Eli Lilly (Nieuwegein, the Netherlands). [G<sup>-3</sup>H]vinblastine sulfate in ethanol was purchased from Amersham International (Little Chalfont, UK). Labeled and unlabeled vinblastine were dissolved in ethanol, dried under nitrogen at 37°C, redissolved at 0.2 mg/ml in 5% dextrose, and administered at 250 kBq/animal. Deacetylvinblastine sulfate and vintripot melathene sulfonate were obtained from the Medgenix Group (Fleurus, Belgium). Hypnorm (fentanyl 0.2 mg/ml, fluanisone 10 mg/ml) and Dormicin (midazolam 5 mg/ml) originated from Janssen Pharmaceuticals B.V. ( Tilburg, the Netherlands) and Roche Nederland B.V. (Mijdrecht, the Netherlands), respectively. BSA was purchased from Organon Teknika (Boxtel, the Netherlands). All other chemicals (E. Merck, Darmstadt, Germany) were of analytical or Lichrosolv gradient grade. Diethyl ether was distilled once before use; the other chemicals were used as supplied. Water was purified by the Milli-Q Plus system (Millipore, Milford, MA). Blank human plasma was obtained from healthy donors.

Study Design. Mice were anesthetized by i.p. administration of 5 to 7 ml/kg b.wt. of the anesthetic solution (Hypnorm/Dormicin/water, 1:1:2, v/v/v). After opening of the abdominal cavity, the common bile duct was ligated. The gallbladder was then cannulated using polyethylene tubing (Portex Ltd, Hythe, UK) with an inner diameter of 0.28 mm. The cannula was ligated to the gallbladder. Dose levels of 5 mg/kg of doxorubicin·HCl or 1 mg/kg of [G<sup>-3</sup>H]vinblastine sulfate were injected into a tail vein. Bile was collected for up to 90 min after drug administration. The temperature of the animals was monitored with a rectal probe and maintained at 36 ± 1°C using an electric heating pad and an infrared lamp. The exposed abdominal tissues were moistened with saline to prevent tissue dehydration. Additional anesthesia (approximately 30 μl) was administered directly into the opening of the abdominal cavity, if required. At the end of the 90-min period, blood samples were taken from the axillary plexus and the contents of small intestine, cecum, and colon were separately collected. Blood samples (collected in heparinized tubes) were centrifuged (10 min, 2100g, 4°C) to separate the plasma fraction, which was stored for analysis. All bile samples were diluted with 1 ml of blank human plasma. The intestinal contents collected in experiments with doxorubicin and vinblastine were homogenized with a Polytron tissue homogenizer (Kinematica AG, Littau, Switzerland) in 2 to 3 ml of 4% (w/v) BSA in water and 2 to 3 ml of blank human plasma, respectively. All biological specimens were stored at −20°C until analysis.

Drug Analysis. Doxorubicin and its metabolites doxorubicinol, 7-deoxydoxorubicinolone, and 7-deoxydoxorubicinone were quantified by a validated reversed-phase high-performance liquid chromatographic fluorescence assay with liquid-liquid extraction using chloroform/1-propanol for sample cleanup (van Asperen et al., 1998). The analysis of vinblastine and its metabolite deacetylvinblastine was performed as described previously (van Tellingen et al., 1993; van Asperen et al., 1996). Briefly, the compounds were extracted from the biological matrices with diethyl ether. The organic phase was dried, reconstituted in acetonitrile, and subjected to ion exchange normal phase high-performance liquid chromatography with fluorescence detection. Radioactivity was determined in diluted bile and in homogenates of intestinal contents using aliquots of 50 and 200 μl, respectively. After adding 5 ml of Ultima Gold scintillation liquid (Packard Instrument Co., Meriden, CT) and mixing, radioactivity was counted in a Tri-Carb Series 4000 Minaxi model B4430 liquid scintillation counter (Packard Instrument Co.) with quench correction by external standardization.

Statistical Analysis. Significant differences between wild-type and mdr1a<sup>−/−</sup> mice were assessed by the Mann-Whitney U test (two-tailed). A P < .05 was regarded as significant.

Results

The absence of mdr1a P-glycoprotein had a profound effect on the biliary secretion of doxorubicin (Table 1). More than 13% of the administered dose was recovered as unchanged drug in the bile of wild-type mice, whereas this was reduced to only 2.4% in mdr1a<sup>−/−</sup> mice. In addition, a substantial fraction of the dose (approximately 10%), which almost exclusively consisted of the parent drug, was secreted via the intestinal wall. The intestinal secretion was similar in both types of mice. The plasma concentrations of doxorubicin observed in these experiments and those observed in our previous study with noncannulated mice (van Asperen et al., 1999) were comparable. The finding of a significantly higher intestinal secretion of 7-deoxydoxorubicinolone seems puzzling, in particular, because the secretion of the other metabolites doxorubicinol and 7-deoxydoxorubicinone was not different. However, this may be a chance finding because there was one animal in the mdr1a<sup>−/−</sup> series with both a relatively high plasma level and intestinal contents, and the difference lost significance when this animal was omitted from the analysis (P = 0.073).

Only minor amounts of both vinblastine and its metabolite deacetylvinblastine were recovered in the bile of wild-type and mdr1a<sup>−/−</sup> mice (Table 2). The biliary secretion of these compounds was significantly lower in the absence of mdr1a P-glycoprotein. About 25 to 30% of the dose was secreted as unknown <sup>3</sup>H-labeled breakdown products in the bile, and this was not significantly different between both types of mice. Direct secretion via the gut wall was primarily observed in the small intestine. The intestinal contents of mdr1a<sup>−/−</sup> mice contained 2-fold lower amounts of unchanged drug. The plasma levels of vinblastine were in the same range as those previously observed in noncannulated animals (van Asperen et al., 1996).

Discussion

This study comparing results in wild-type and mdr1a<sup>−/−</sup> mice shows the drug-dependent effect of mdr1a P-glycoprotein on the secretion of substrate agents via the bile and the intestinal wall. Discrimination between each of these secretion pathways was achieved by using mice with a cannulated gallbladder. Drugs excreted in the bile were not able to re-enter the body by enterohepatic cycling.
and drug recovered in the intestinal lumen could only have reached this site by direct secretion through the intestinal wall. Selective analytical methods allowed the separate quantification of unchanged drug and metabolites.

The experiments clearly demonstrate that mdr1a P-glycoprotein significantly contributes to the biliary secretion of doxorubicin, but its absence does not result in a diminished direct secretion of this drug through the intestinal wall. In contrast, the biliary secretion of unchanged vinblastine was already minimal in wild-type mice, whereas its intestinal secretion was substantially reduced in the absence of mdr1a P-glycoprotein. Important, additional information was obtained by integration of the present results of the gallbladder cannulation experiments with previous data of urinary and fecal excretion studies, which will be discussed below. The animals in the present study were anesthetized during the experiment until the moment of sacrifice and the anesthetics may affect the fate of the investigated drugs. However, both in vinblastine and in doxorubicin treated animals the plasma levels at the end of the experiment were similar to those in awake animals in our previous studies (van Asperen et al., 1996, 1999). The experiment was terminated 90 min after the start before a deteriorated condition of the animal and depletion of bile salts could have been able to affect the bile flow.

Within 90 min after i.v. administration of vinblastine, more than 25% of the administered dose was recovered as unknown 3H-labeled breakdown products in the bile of both types of mice. This result is in line with previous data showing that fecal excretion of metabolic breakdown products is an important pathway of elimination for vinblastine with 70 to 80% of the radioactivity recovered in the feces within 0 to 48 h (van Tellingen et al., 1993; van Asperen et al., 1996). This result was also in line with another report on biliary excretion in the rat although the fraction of unchanged drug in mice appears to be much lower (Zhou et al., 1990). The cumulative fecal excretion of unchanged vinblastine (within 48 h after drug administration) was only approximately 20 and 10% of the dose in wild-type and mdr1a(−/−) mice, respectively (van Asperen et al., 1996). Although the present results give only information up to 90 min after drug administration, they suggest that the diminished fecal excretion of vinblastine in mdr1a(−/−) mice mainly results from a decreased secretion through the intestinal wall. The minor biliary secretion of unchanged drug implies that an increased reuptake of this drug fraction from the intestinal lumen in mdr1a(−/−) mice would hardly affect the total fecal excretion. This result is in marked contrast to the results with paclitaxel (Sparreboom et al., 1997). Whereas the biliary secretion of i.v. administered paclitaxel was unaltered in mdr1a(−/−) mice, the fecal excretion decreased from 40% in wild-type to 2% in mdr1a(−/−) mice. This effect could be explained by the almost complete re-absorption of paclitaxel from the gut. The intestinal secretion of vinblastine, however, was not completely abolished in mdr1a(−/−) mice. The mechanism responsible for this intestinal secretion is unknown. The mdr1b P-glycoprotein is unlikely to play a role in this transport, because it could not be detected in the intestines of mdr1a(−/−) mice (Schinkel et al., 1994).

The results for doxorubicin were different. Approximately 10% of the dose was recovered as unchanged drug in the intestinal contents of both wild-type and mdr1a(−/−) mice with a cannulated gallbladder. This indicates that whereas direct intestinal secretion also appears to be an important route of elimination for doxorubicin, P-glycoprotein does not seem to play an important role in this process. At least in mdr1a(−/−) mice, intestinal secretion is mediated by mechanisms other than transport by P-glycoprotein. In contrast, the absence of mdr1a P-glycoprotein significantly decreased the biliary secretion of doxorubicin. Only a small fraction of the dose, which may have been transported by mdr1b P-glycoprotein, was recovered in the bile of mdr1a(−/−) mice. The mdr1b gene is expressed in the liver and

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Doxorubicin</th>
<th>Doxorubicinol</th>
<th>7-Deoxydoxorubicinolone</th>
<th>7-Deoxydoxorubicinone</th>
</tr>
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<tr>
<td></td>
<td>Wild-type</td>
<td>mdr1a(−/−)</td>
<td>Wild-type</td>
<td>mdr1a(−/−)</td>
</tr>
<tr>
<td>Plasma</td>
<td>150 ± 22</td>
<td>166 ± 23</td>
<td>21.9 ± 4.6</td>
<td>23.0 ± 2.6</td>
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<tr>
<td>Bile</td>
<td>13.3 ± 1.7</td>
<td>2.4 ± 0.3</td>
<td>0.53 ± 0.09</td>
<td>0.20 ± 0.05</td>
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<tr>
<td>Contents of:</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>small intestine</td>
<td>10.0 ± 0.6</td>
<td>9.6 ± 0.4</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>cecum</td>
<td>0.11 ± 0.02</td>
<td>0.16 ± 0.03</td>
<td>0.18 ± 0.04</td>
<td>0.66 ± 0.38</td>
</tr>
<tr>
<td>colon</td>
<td>0.34 ± 0.15</td>
<td>0.25 ± 0.08</td>
<td>0.04 ± 0.01</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>total intestine</td>
<td>10.5 ± 0.5</td>
<td>10.0 ± 0.4</td>
<td>0.06 ± 0.01</td>
<td>0.01 ± 0.00</td>
</tr>
</tbody>
</table>

Data (means ± S.E.) are represented as a percentage of the administered dose (plasma concentration at t = 90 min in nM); *P < .05 versus wild-type mice (n = 7; 2n = 8).

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Deacetylvindicline</th>
<th>Deacetylvindicline</th>
<th>Deacetylvindicline</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>mdr1a(−/−)</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Plasma</td>
<td>31.4 ± 7.1</td>
<td>23.4 ± 4.0</td>
<td>9.1 ± 1.5</td>
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<tr>
<td>Bile</td>
<td>1.1 ± 0.2</td>
<td>0.38 ± 0.06</td>
<td>0.19 ± 0.02</td>
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<td>Contents of:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>small intestine</td>
<td>5.4 ± 0.7</td>
<td>2.8 ± 0.5</td>
<td>0.57 ± 0.04</td>
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<tr>
<td>cecum</td>
<td>1.0 ± 0.1</td>
<td>0.14 ± 0.03</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>colon</td>
<td>0.37 ± 0.06</td>
<td>0.30 ± 0.06</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>total intestine</td>
<td>6.7 ± 0.7</td>
<td>3.3 ± 0.6</td>
<td>0.62 ± 0.04</td>
</tr>
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</table>

Data (means ± S.E.) are represented as a percentage of the administered dose (plasma concentration at t = 90 min in nanograms per milliliter); *P < .05 versus wild-type mice (n = 6 animals per group).

n.a., not available.
increased levels of its product were detected in livers of \( mdr1a(-/-) \) mice (Schinkel et al., 1994). A reduced biliary excretion of doxorubicin is in line with previous reports using isolated perfused rat livers, which showed that the addition of a P-glycoprotein blocker or substrate also caused a marked reduction (Booth et al., 1998; Smit et al., 1998). Despite its pronounced effect on the biliary secretion of unchanged doxorubicin, previous experiments demonstrated that the absence of \( mdr1a \) P-glycoprotein did not result in a reduced cumulative fecal excretion of this compound. Whereas a previous study in rats receiving \(^{14} \text{C}-\)labeled doxorubicin showed that about 65% of the administered radioactivity was recovered in the feces (Arcamone et al., 1984), approximately 4 to 5% of the dose was recovered unchanged in the feces of both wild-type and \( mdr1a(-/-) \) mice within 96 h after i.v. administration of 5 mg/kg of doxorubicin (van Asperen et al., 1999). Hence, a similar fecal excretion of a substrate drug in wild-type and \( mdr1a(-/-) \) mice does not exclude the possibility that \( mdr1a \) P-glycoprotein may play a role in its biliary and/or intestinal secretion.

Furthermore, the present experiments show that within 90 min after administration of doxorubicin about 25 and 12% of the dose was secreted unchanged in bile plus intestinal contents of wild-type and \( mdr1a(-/-) \) mice, respectively. The finding of only 4 to 5% of unchanged drug in the feces suggests that doxorubicin may undergo substantial degradation in the intestinal lumen. Moreover, a recent study in rats showed that a substantial fraction of doxorubicin excreted in bile may be reabsorbed from the gut (Behnia and Boroujerdi, 1998). The identity of the metabolites excreted in the feces is unknown. However, the fact that they are not detected by our assay suggests that these may either be a more polar conjugated species or that the changes in the molecule involve alterations in the basic fluorescent anthracycline ring structure. Overall, only about 25% of the drug was recovered in feces and urine as parent drug or measurable metabolites (van Asperen et al., 1999).

Together with our previous results with paclitaxel (Sparreboom et al., 1997), this study clearly demonstrates the marked differences in pharmacokinetic handling of drugs by P-glycoprotein in vivo. Although these drugs are all good substrates for P-glycoprotein and behave similarly in many in vitro systems, their in vivo fate in the presence or absence of P-glycoprotein at principal drug elimination sites like the intestinal wall and liver varies considerably. This finding needs to be kept in mind when in vitro screening models are being used to assess the clinical usefulness of agents.

References


