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Interactions between P-glycoprotein substrates and other cationic drugs at the hepatic excretory level

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1 In the present study it was tested whether known P-glycoprotein (P-gp) substrates/MDR reversal agents interact with small (type 1) and bulky (type 2) cationic drugs at the level of biliary excretion in the rat isolated perfused liver model (IPRL). The studies were performed with model compounds tri-n-butylmethylammonium (TBuMA) (a relatively small type 1 organic cation), rocuronium (Roc) (a bulky type 2 organic cation) and the classical P-gp substrate doxorubicin (Dox).

2 Inhibitors were given in a 4 fold molar excess to the substrate studied. To minimize an interaction of the substrates at the hepatic uptake level, the competing compounds were added when over 55% to 85% of the administered dose of the model compounds had been removed from the perfusate and taken up by the liver.

3 We found a mutual interaction between TBuMA and procainamidethobromide (PAEB), both type 1 cationic compounds during biliary excretion. Interestingly, type 2 compounds, such as rocuronium, clearly inhibited type 1 cationic drugs as well as Dox secretion into bile, whereas type 1 compounds did not significantly inhibit type 2 drug excretion into bile. The type 1 cations PAEB and TBuMA only moderately inhibited Dox biliary excretion. Dox did not inhibit the biliary excretion of the type 2 agent rocuronium whereas rocuronium reduced Dox biliary excretion by 50% compared to controls.

4 MDR substrates/reversal agents like verapamil, quinine, quinidine and vinblastine strongly reduced both type 1 and type 2 organic cation excretion into bile. Dox secretion into bile was also profoundly reduced by these drugs, vinblastine being the most potent inhibitor in general.

5 The lack of mutual inhibition observed in some combinations of substrates may indicate that major differences in affinity of the substrates for a single excretory system exist. Alternatively, multiple organic cation transport systems with separate substrate specificities may be involved in the biliary excretion of amphiphilic drugs. Furthermore, the present study revealed a clear positive correlation between the lipophilicity of the potential inhibitors studied and their respective inhibitory activity on the biliary excretion of the model drugs investigated.

6 Our data are compatible with a potential involvement of P-glycoprotein in the hepatobiliary excretion of doxorubicin as well as of some type 1 and type 2 organic cations. Furthermore we postulate that the hydrophobic properties of the amphiphilic cationic drugs studied play a crucial role in the accommodation of these agents by P-glycoprotein and/or other potential cationic drug carrier proteins in the canalicular membrane.

Keywords: Hepatobiliary transport; cationic drugs; drug interaction; P-glycoprotein; lipophilicity

Introduction

The liver plays a central role in the elimination of amphiphilic drugs from the body (Oude Elferink *et al*., 1995; Meijer et al., 1997). The mechanisms involved in the hepatic uptake of organic cations have been studied mainly in the rat *in vivo*, in rat isolated perfused livers (Meijer *et al*., 1976; Ballet *et al*., 1987; Stapf *et al*., 1994), in isolated hepatocytes (Okudaira *et al*., 1992; Miyauchi *et al*., 1993; Nakamura *et al*., 1994) and basolateral plasma membrane vesicle studies (Moseley *et al*., 1990; 1992a,b; 1996; McKinney & Hosford, 1992). The overall picture emerging from these studies is that multiple mechanisms exist for the hepatic uptake of organic cations. Relatively small monovalent cationic compounds like tri-n-butylmethylammonium (TBuMA), are taken up via the so-called type 1 transport system (Steen *et al*., 1991). Interestingly, by use of a cDNA library from rat kidney, a gene has recently been cloned that encodes a transmembrane protein capable of transporting the type 1 cation tetraethylammonium (TEA). It appeared that this protein is also highly expressed in the liver and most likely plays a role in the hepatic uptake of type 1 organic cationic compounds Gründemann *et al*., 1994; Martel *et al*., 1996).

Larger and more bulky cations (the type 2 category) have one or two positively charged nitrogen groups included in or situated close to hydrophobic moieties in the molecule. With regard to hepatic uptake, these type 2 compounds behave differently from the type 1 cations (Meijer *et al*., 1990; 1991). Certain cardiac glycosides and bile acids are potent inhibitors of hepatic type 2 cation uptake (Meijer *et al*., 1971; 1991; Okudaira *et al*., 1992), but do not affect the uptake of type 1 organic cationic compounds. Recently Bossuyt *et al*. (1996a,b) showed that the organic anion transporting polypeptide, encoded by the *oatp* gene (Kullak-Ublick *et al*., 1995), catalyzes organic anion as well as cardiac glycoside transport, but also the transport of bulky type 2 cationic drugs. Therefore, organic anion transporting polypeptide is a likely candidate transporter that may be involved in the hepatic uptake of type 2 organic cations.

At the level of biliary excretion of organic cations, little detailed information on transport mechanisms is presently available. Evidence has been obtained from canalicular membrane vesicle studies, that P-glycoprotein might be

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involved in biliary excretion of amphiphilic antineoplastic drugs (Kamimoto *et al*., 1989). Extensive reviews on the potential role of P-glycoprotein in the biliary excretion processes have been published recently by us (Oude Elferink *et al*., 1995; Meijer *et al*., 1997). In short, several papers have described the inhibitory effects of multidrug resistance (MDR) substrates/reversal agents like e.g. cyclosporin A (CsA) on the excretion of cationic drugs into bile in the rat perfused liver model (Watanabe *et al*., 1992; Speeg *et al*., 1992; Thalhammer *et al*., 1994; Speeg & Maldonado, 1994; Stapf *et al*., 1994). These studies provided at least indirect evidence for the involvement of (P-glycoprotein P-gp) in the biliary excretion of organic cationic compounds in the rat liver.

Müller *et al*., (1994) showed ATP dependent transport of amphiphilic cationic drugs such as azopentyl-deoxy-ajmalinium (APDA) (a type 2 cation) in plasma membrane vesicles that were derived from a rat mdr1b P-gp expression system. They also showed that isolated canalicular plasma membrane vesicles from rat liver (cLPM) harbored an ATP-dependent transport system resembling the characteristics of the expressed mdr1b P-gp (Müller *et al*., 1994). In contrast, Moseley *et al*., (1992a; 1996) presented data indicating the existence of an ATP-independent cation:H^+-exchange mechanism also using canalicular liver plasma membrane vesicles. It was found that some type 2 cations inhibit the H^+-dependent type 1 cation transport in these vesicles. This is analogous to the situation at the sinusoidal level where type 2 cationic compounds inhibit the uptake of type 1 cationic agents but not *vice versa* (Steen *et al*., 1992). Moseley *et al*., (1996) concluded that transport in canalicular membrane vesicles of type 1 cations is functionally different from P-glycoprotein mediated transport, since no ATP-dependence was detected and cation transport could not be inhibited by the P-gp substrate daunomycin.

Since at present no systematic studies have been performed in the intact liver to investigate organic cation transport at the bile canalicular level, we performed a large series of experiments to characterize cationic drug excretion into bile. For this purpose we studied the classical P-gp substrate doxorubicin and measured the influence of concomitantly administered organic cations, other P-gp substrates and some MDR reversal agents on the biliary excretion rate. To establish if mutual inhibition at the bile canalicular level occurs we also studied the influence of P-gp substrates and reversal agents on the biliary excretion rate of type 1 and type 2 organic cations. In addition, the observed inhibitory patterns were related to the relative lipophilicity of the agents.

Methods

The isolated perfused liver technique of the rat

A liver perfusion technique was used as described earlier (Meijer *et al*., 1981) with slight modifications. Briefly, rats deprived of food were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.). After cannulation of the portal vein, and the superior vena cava and the common bile duct, the liver was excised and placed in the perfusion apparatus. The recirculating medium consisted of Krebs bicarbonate buffer supplemented with 1% (w/v) bovine serum albumin (BSA) and was constantly gassed with 95% O_2 and 5% CO_2. The perfusate flow was maintained at 35 m min^{-1} at a hydrostatic pressure of 12 cm to assure sufficient oxygen supply. The pH was monitored on line and ranged between 7.35 and 7.45. The cabin temperature was kept at 38°C. To obtain a constant bile flow and to avoid bile salt depletion, taurocholate was infused at a rate of 15 μmol h^{-1}. After a 30 min recovery period following the surgical procedure, 1.2 μmol ($C_{0,1} \sim 10$ μM) of the model compounds studied were added to the perfusion medium. The second (inhibitory) compound was added in a 4 fold higher concentration (unless otherwise noted) after 20 or 30 min, a time point at which 50 to 90% of the dose of the studied compound had been taken up by the liver. Thus, interactions at the hepatic uptake level were expected to be minimal. Furthermore, when hepatic uptake of the model compound studied was also inhibited, the liver to perfusate and the bile to liver concentration ratios were calculated and compared. Inhibition at the bile secretory level is considered to occur if bile secretion of the model compound is decreased leading to a decreased bile to liver ratio and that its decrease is larger than that between liver and perfusate. The inhibitory effect on the biliary excretion rate of the added compounds was calculated from the cumulative biliary excretion following the addition of the inhibitory agents compared to controls.

The dose regimen chosen was based on previously estimated K_m values for hepatobiliary transport of organic cations (Neef *et al*., 1984) and the values for transport of MDR substrates in P-glycoprotein expressing cell systems (Gottesman & Pastan, 1993; Ghauharali *et al*., 1996; Spoelstra *et al*., 1996).

The compounds studied were administered in a dose of 1.2 μmol leading to an initial perfusate concentration of 10 μM. Assuming that the agents reach intracellular cytoplasmic concentrations of about 3 times the perfusate concentration, through facilitated diffusion and equilibration according to the hepatocyte membrane potential (Meijer *et al*., 1990), a significant occupation of binding sites on the canalicular carriers can be expected without a complete saturation of the particular transport systems. The latter is confirmed by the observed excretion rate patterns for all of the model compounds in the present study. Potential inhibitory compounds were administered in a 4 times higher molar dose compared with the agents studied, unless stated otherwise. All of the inhibitors (potential competing compounds) are known to be rapidly taken up in the liver.

Quinine, quinidine, verapamil and digoxin were dissolved in dimethylsulphoxide (DMSO). DMSO content in the perfusate never exceeded 0.1% (v/v). All other compounds were dissolved in Krebs buffer or directly in demineralized water.

Animals

Male Wistar rats (Harlan Institute, The Netherlands), weighing 250–290 g, served as liver donors in the perfusion studies described. All animals were maintained at a controlled temperature condition and were fed standard chow (RHM, Hope Farms, Woerden, The Netherlands) and obtained water *ad libitum* on a 12/12 h light/dark cycle.

Drug analysis

Bile secretion of unchanged doxorubicin was analysed and separated from its metabolites by use of fluorescence high performance liquid chromatography (h.p.l.c.) according to the method of Beijnen *et al*. (1991) with slight modifications. A Hypersil ODS column (Shandon Sci. Ltd, Astmoor, U.K.) (100 × 2 mm, particle size 5 μm) and an eluent containing acetonitril:tetrahydrofuran:phosphate buffer (50 mM, pH 2.7) = 8:12:80 was used. All the eluent components were filtered through a 0.45 μm pore size (phosphate buffer) or a 0.5 μm (organic fluids) filter before use. Eluent flow was 0.4 ml min^{-1}. Fluorescence was measured with a Waters 486

fluorimeter at 506/594 nm excitation/emission wave length and monitored by use of a Kipp recording apparatus (Kipp & Sons, The Netherlands).

Rocuronium bile contents were analysed with a fluorimetric method as described previously (Paanakker *et al*., 1987). [^{3}H]-TBuMA contents in perfusate and bile were determined by scintilation counting on a Beckman LS1800 Liquid scintillation counter, with Pico Fluor solution (Packard, Groningen, The Netherlands).

Partition coefficients

We investigated the (relative) lipophilicity of the cationic compounds studied by determining the *n*-octanol-aqueous phase partition coefficient. As a physiological aqueous phase we used Krebs-Henseleit solutions from which carbon dioxide and sodium bicarbonate were omitted (Paanakker *et al*., 1987). The reason for this omission is that CO_2 is extracted in the octanol phase changing the pH of the aqueous as well as the polarity of the lipoid phase during the partition procedure. The pH of this physiological salt solution, that still contains an inorganic phosphate buffer, was adjusted to pH 7.4 with sodium hydroxide. The compounds were dissolved in equal volumes of *n*-octanol and Krebs solution (2.5 ml). The two phases were mixed on a whirl mixer for 1 min and shaken on a rotating tumbler (53 r.p.m.) for 2 h at 20°C. The layers were separated by centrifugation at 2500 r.p.m. and the samples were analysed by fluorimetric or u.v. measurements.

Chemicals

Doxorubicin hydrochloride (Adriamycin) was purchased from Farmitalia (Carlo Erba, Italy). Rocuronium was from Organon Technika (Turnhout, Belgium). (+)-Tubocurarine chloride was from Burroughs Wellcome & Co. (London, U.K.). Tri-n-butylmethylammonium (TBuMA) was synthesized in our laboratory, according to the procedures described by Neef *et al*. (1983). Vinblastine sulphate (Velbe) and vincristine sulphate (Oncovin) were purchased from Eli Lilly Nederland B.V. (Amsterdam, The Netherlands). Quinine hydrochloride, quinidine hydrochloride, verapamil hydrochloride, digoxin were from Sigma (St. Louis, MO, U.S.A.). Taurocholate was from Fluka Chemie AG (Buchs, Switzerland). Procainamidethobromide (PAEB) was kindly donated by E.K. Squibb & Sons Inc. (Princeton, NJ, U.S.A.).

Tetrahydrofuran was purchased from Janssen Chimica (Gell, Belgium). H.p.l.c.-grade acetonitrile was obtained from Labscan (Dublin, Ireland). All other chemicals were from Merck (Darmstadt, Germany).

Statistical analysis

Statistical comparisons were made with Student's *t* test (two-tail). Level of significance was set at $P<0.05$. Linear correlation tests were performed by use of linear regression analysis (least square analysis) to select the best fitting lines.

Results

Interactions at the bile secretory level between MDR substrates and type 1/type 2 cationic drugs were investigated with doxorubicin, TBuMA and rocuronium as model substrates, respectively. We attempted to demonstrate interactions at the biliary excretory level without major influences of uptake inhibition.

In the perfusion studies the uptake of the cationic drugs into the liver was relatively rapid. At the time of addition of the potential inhibitors over 85% of the administered dose of rocuronium and doxorubicin was already taken up by the liver, for TBuMA this was about 55%. Hence potential inhibitory compounds could be added at a time when a considerable amount of the particular substrate had already been taken up

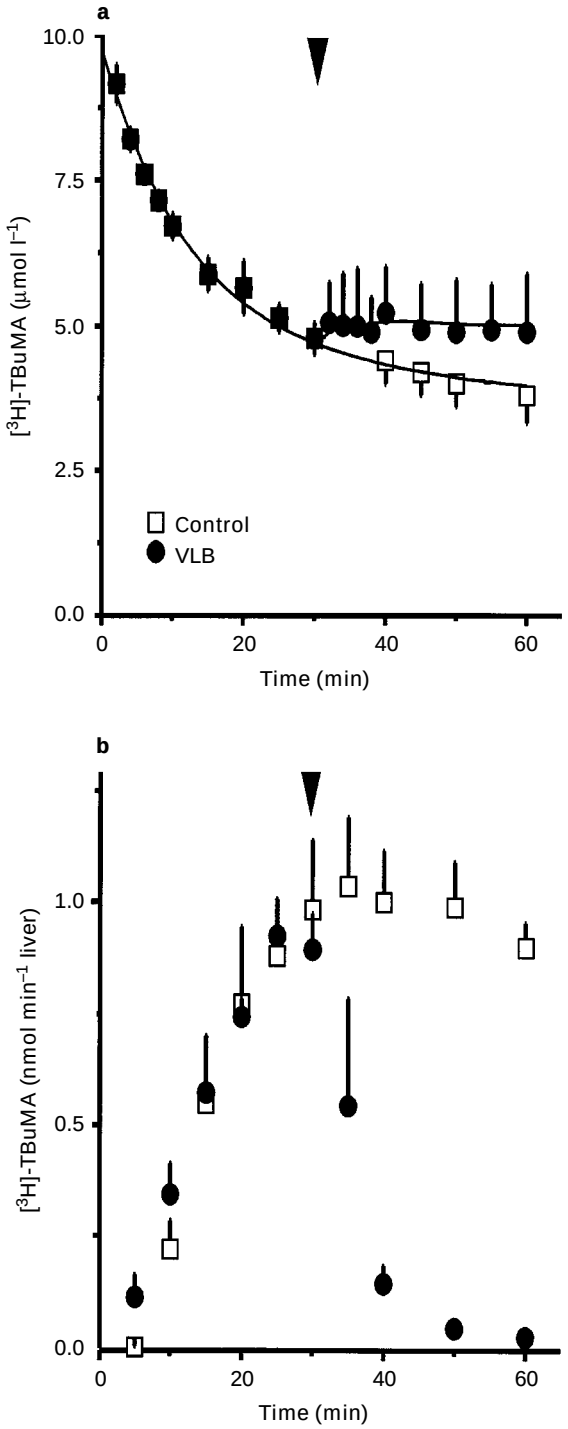


Figure 1 The perfusate concentration versus time curve (a) and the biliary excretion rate versus time curve (b) of [^{3}H]-TBuMA in the rat isolated perfused liver. TBuMA was administered as a bolus of 1.2 μmol at t = 0 min. ($C_0 \sim 10$ μM). Data are the mean of three to six independent experiments; vertical lines show s.e.mean. At the designated time point indicated by the arrows the potential inhibitor (vinblastine (VLB, 4.8 μmol)) was added to the perfusate.

by the liver. Figure 1 shows that upon addition of the inhibitor further plasma decay of TBuMA seemed to be partly inhibited (a). However, the inhibition of biliary secretion of TBuMA was much more profound (b). In addition, the calculated liver to perfusate and bile to liver concentration ratios of TBuMA suggest that the bile secretion, particularly was inhibited and confirmed the latter conclusion for this compound. At the time of addition of the potential inhibitors over 80% of the administered dose of doxorubicin had been taken up by the liver, similar to rocuronium. Therefore the addition procedure was chosen did indeed minimizes the impact of interactions at the level of hepatic uptake.

Doxorubicin excretion

The effects of several inhibitory compounds on doxorubicin (Dox) biliary excretion rate in the rat isolated perfused liver is shown in Figure 2. Dox levels in the perfusate were

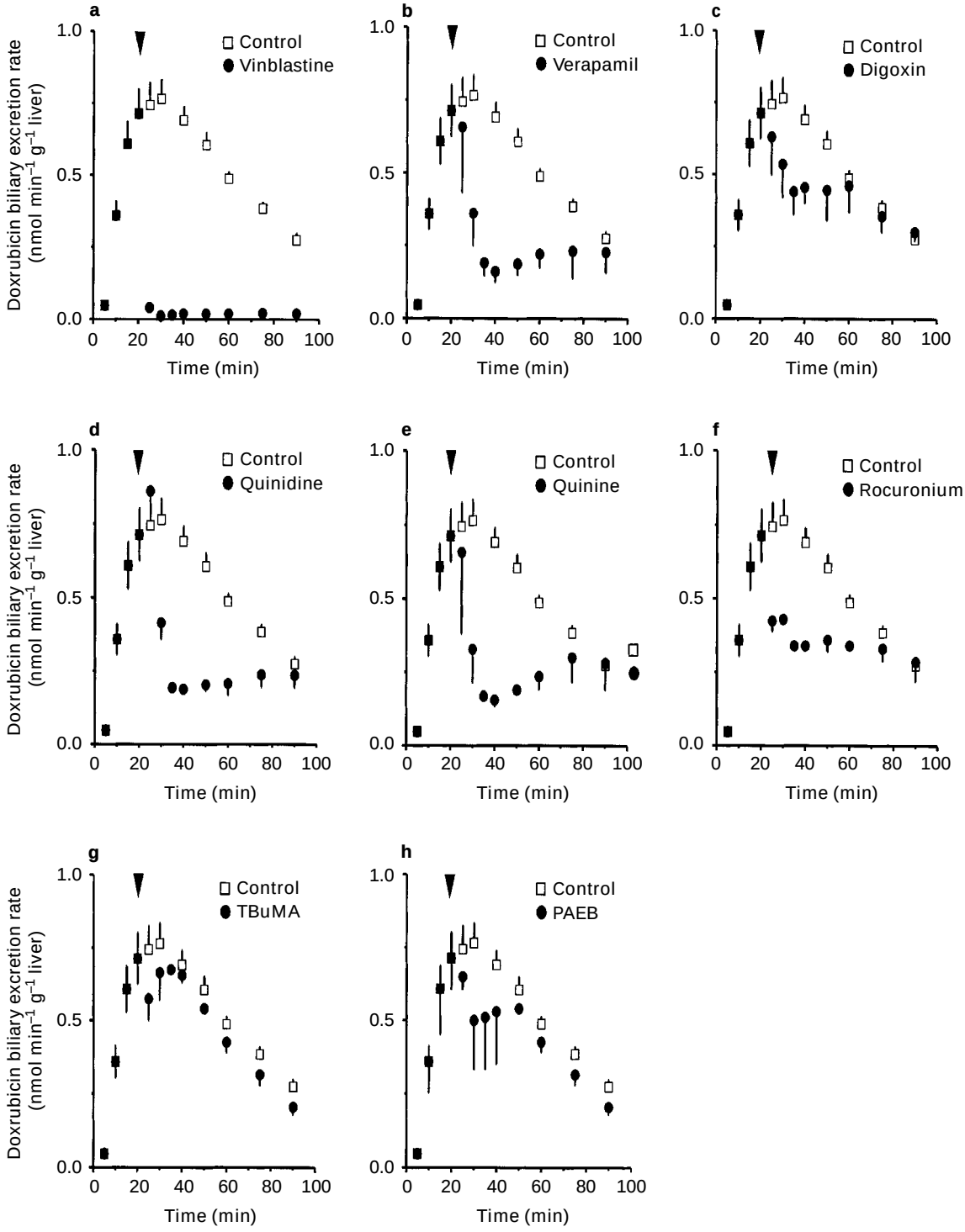


Figure 2 The biliary excretion of doxorubicin in the rat isolated perfused liver. Doxorubicin was administered as a bolus of 1.2 μmol at t = 0 min. ($C_0 \sim 10$ μM). Potential inhibitors ((a) vinblastine, (b) verapamil, (c) digoxin, (d) quinidine, (e) quinine, (f) rocuronium, (g) TBuMA, (h) PAEB) were given as a bolus of 4.8 μmol at the time point indicated by the arrow. Data are the mean of six (control) and at least three independent inhibition experiments; vertical lines show s.e.mean.

simultaneously recorded. Plasma disappearance following the administration of potential inhibitor compounds was only slightly affected, resulting in a somewhat slower disappearance compared to control (between t = 20 min and 60 min, see also Figure 1). The hepatic uptake of Dox was rapid: after 20 min roughly 90% of the dose was removed from the perfusate by the liver.

The addition of vinblastine at t = 20 min, in an amount at four times the molar dose of doxorubicin, strongly reduced the maximal Dox biliary excretion rate to maximally 3% of the control value (Figure 2a). It was noteworthy that, after the addition of vinblastine to the perfusate (40 μM), in all cases the bile flow increased approximately two fold for about 20 min returning to the initial value at t = 60 min (data not shown). Addition of verapamil also resulted in a marked reduction in the excretion of Dox into bile-excretion rate was decreased to about 25% of the control (Figure 2b). Upon addition of the cardiac glycoside digoxin only a modest reduction in the

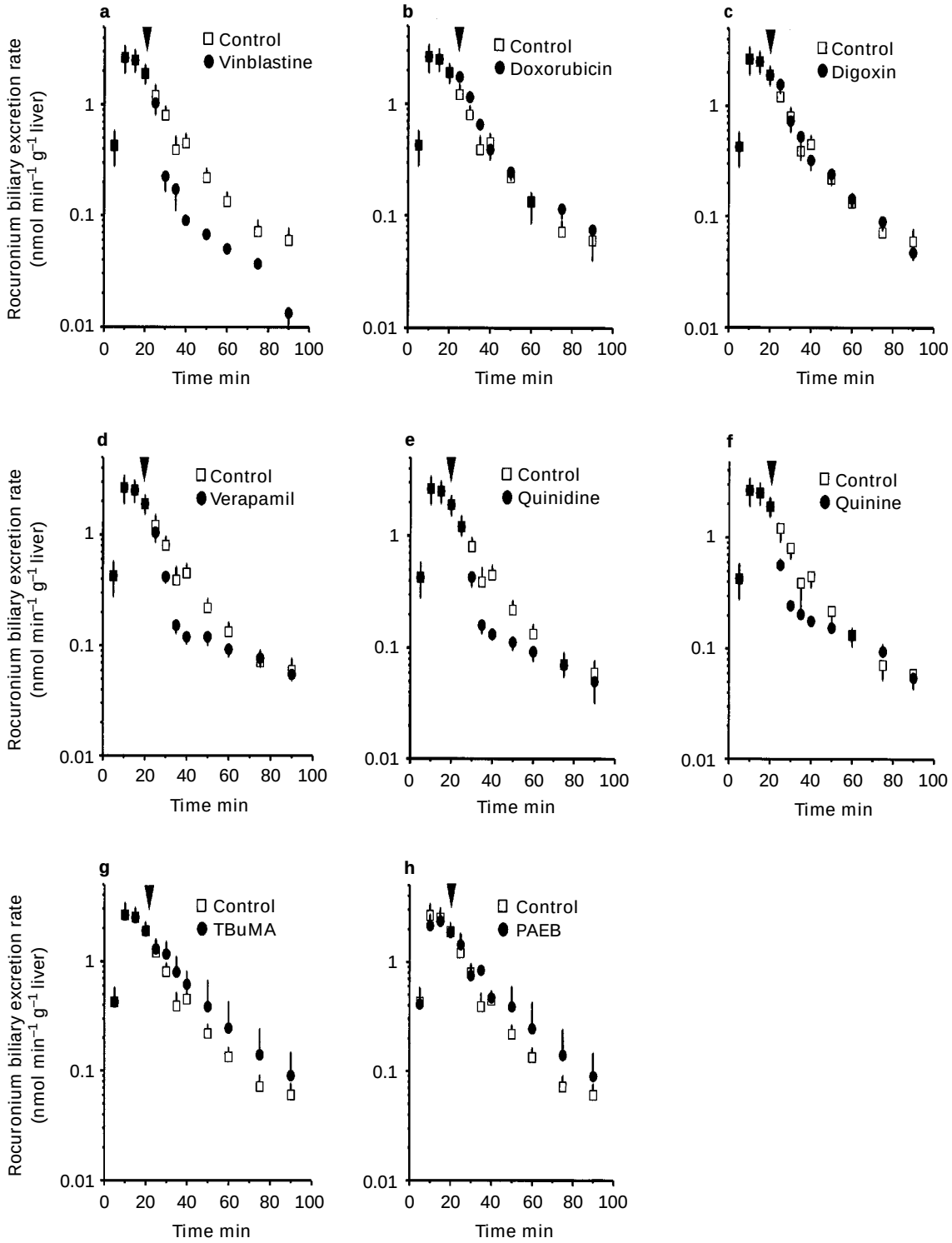


Figure 3 The biliary excretion of rocuronium in the rat isolated perfused liver. Rocuronium was administered as a bolus of 1.2 μmol at t = 0 min. ($C_0 \sim 10$ μM). Potential inhibitors ((a) vinblastine, (b) doxorubicin, (c) digoxin, (d) verapamil, (e) quinidine (f) quinine, (g) TBuMA and (h) PAEB) were given as a bolus of 4.8 μmol at the time point indicated by the arrow. Data are the mean of six (control) and at least three independent inhibition experiments; vertical lines show s.e.mean.

biliary excretion rate of Dox to 65% of the control value was found (Figure 2c). Quinidine and quinine reduced the maximal Dox biliary excretion rate to 29% and 25% respectively, of control (Figure 2d,e).

The type 2 steroidal cation rocuronium also inhibited the biliary excretion rate of Dox to maximally 50% of the control value, (Figure 2f), whereas the addition of the type 1 compound TBuMA had virtually no effect on the biliary excretion rate of Dox (Figure 2g). After addition of procainamide ethobromide (PAEB) the maximal Dox biliary excretion rate was modestly reduced to 75% of the control (Figure 2h).

Rocuronium excretion

The effects of the compounds mentioned above on the biliary excretion rate of the type 2 model cation rocuronium (Roc) is shown in Figure 3. Upon addition of vinblastine, the maximal

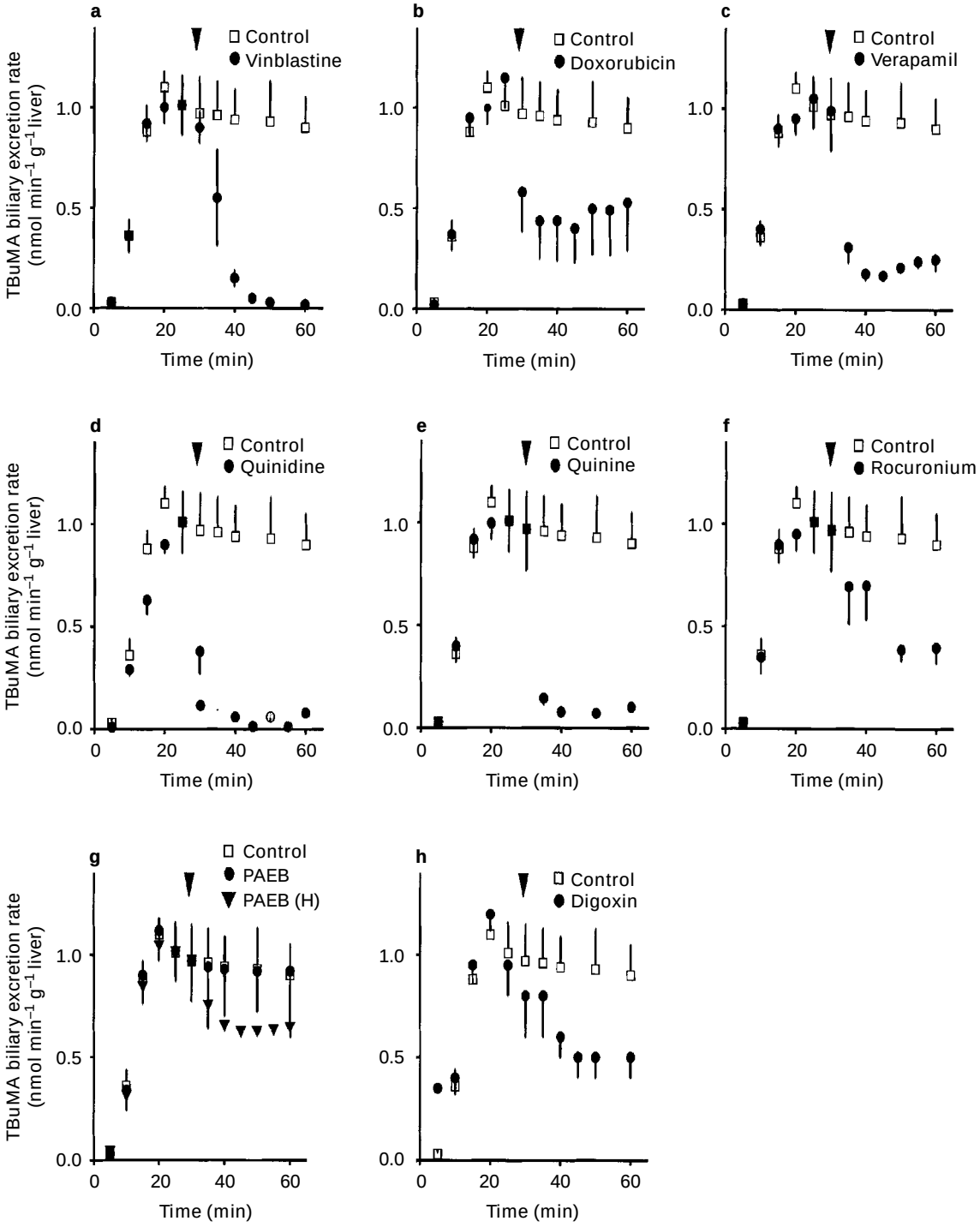


Figure 4 The biliary excretion of TBuMA in the rat isolated perfused liver. TBuMA was administered as a bolus of 1.2 μmol at t = 0 min. ($C_0 \sim 10$ μM). Potential inhibitors ((a) vinblastine, (b) doxorubicin, (c) verapamil, (d) quinidine, (e) quinine, (f) rocuronium, (g) PAEB and (h) digoxin) were given as a bolus of 4.8 μmol at the time point indicated by the arrow (with the exception of doxorubicin (12.2 μmol, b) and PAEB (g, 4.8 μmol and 4.8 μmol (H)). Data are the mean of six (control) and at least three independent inhibition experiments; vertical lines show s.e.mean.

biliary excretion rate of rocuronium was reduced to about 20% of the control value (Figure 3a). Upon addition of doxorubicin no significant effect could be shown on the biliary excretion rate of rocuronium (Figure 3b), whereas the administration of verapamil lead to a decrease of the maximal biliary excretion rate to about 25% of control (Figure 3d).

Digoxin completely blocked the residual hepatic uptake of Roc following the administration of the cardiac glycoside, which is in line with previous findings (Steen *et al*., 1992) (data not shown). However, no significant inhibition could be observed at the biliary excretion level (Figure 3c). Following the addition of quinidine and quinine the maximal biliary excretion rate of rocuronium decreased to 29% and 40% respectively of the control values (Figure 3f). The type 1 cationic compounds TBuMA and PAEB showed no effect on either the plasma decay or the biliary excretion rate of rocuronium (Figure 3g,h).

TBuMA excretion

Vinblastine (at 4 times the molar dose of TBuMA) profoundly reduced the biliary excretion rate of TBuMA to less than 10% of the control value (Figure 4a). A ten times molar dose of Dox, compared to TBuMA, resulted in a reduction of the biliary excretion rate of TBuMA to about 50% of the control value (Figure 4b). After addition of verapamil the maximal biliary excretion rate of TBuMA was decreased to about 16% of the control value (Figure 4c). The addition of quinidine or quinine also resulted in a decrease in the maximal biliary excretion rate of TBuMA to about 30% of control (Figure 4d,e). Digoxin reduced biliary TBuMA output to 60% of the control (Figure 4f).

The type 1 compound PAEB at a 4 fold molar dose did not affect the TBuMA biliary excretion (Figure 4g, PAEB), while a 40 fold molar dose inhibited the biliary excretion rate of TBuMA to about 55% of control value (Figure, 4g, PAEB (H)).

Since the chemical structure and also the related physico-chemical properties of the drugs studied vary, we investigated the (relative) lipophilicity of compounds, expressed as the $\log P_{octanol/water}$ value (Table 1). The relation between the relative lipophilicity of the potential inhibitory compounds and their inhibitory effect on organic cation transport at the canalicular excretion level was subsequently analysed. Table 2 shows the cumulative biliary excretion values obtained, which were calculated from the amount of substrate excreted into bile following addition of a potential inhibitor. We found a clear (positive) linear correlation between the maximal reduction of Dox ($r=0.82$, $P=0.012$), Roc ($r=0.9$, $P=0.002$) and TBuMA ($r=0.86$, $P=0.0064$) biliary excretion and the LogP values of the inhibitors studied (Figure 5).

Table 1 Octanol-Krebs partition coefficient ($P_{Oct/Krebs}$) values of the investigated substrates and of the potential inhibitors studied

No	*Compound*	*log ($P_{Oct/Krebs}$)*
1	Vinblastine	2.04
2	Digoxin	0.79
3	Quinidine	0.71
4	Quinine	0.65
5	Verapamil	0.58
6	Recuronium	−0.01
7	Doxorubicin	−0.80
8	PAEB	−0.85
9	TBuMA	−0.92

Values are expressed as the log P-value.

Table 2 Biliary output of cationic drugs as influenced by P-glycoprotein substrates and other cationic or neutral compounds

Inhibitor	*Dox (294)*	*Roc (176)*	*TBuMA (201)*
1. Quinine	53*	64*	15*
2. Quinidine	54*	72*	24*
3. Verapamil	49*	69*	31*
4. Digoxin	82^{NS}	107^{NS}	55*
5. Rocuronium	67*	–	58*
6. Vinblastine	8*	57*	8*
7. TBuMA	88^{NS}	139*	–
8. PAEB	82^{NS}	130^{NS}	73*
9. Doxorubicin	–	125^{NS}	50*

The percentages depicted are calculated from the cumulative bilary output levels following the addition of the indicated agents compared to controls (100% values are shown in parentheses (nmol)). $P<0.05$; NS, not statistically different; $n=3$ to 6.

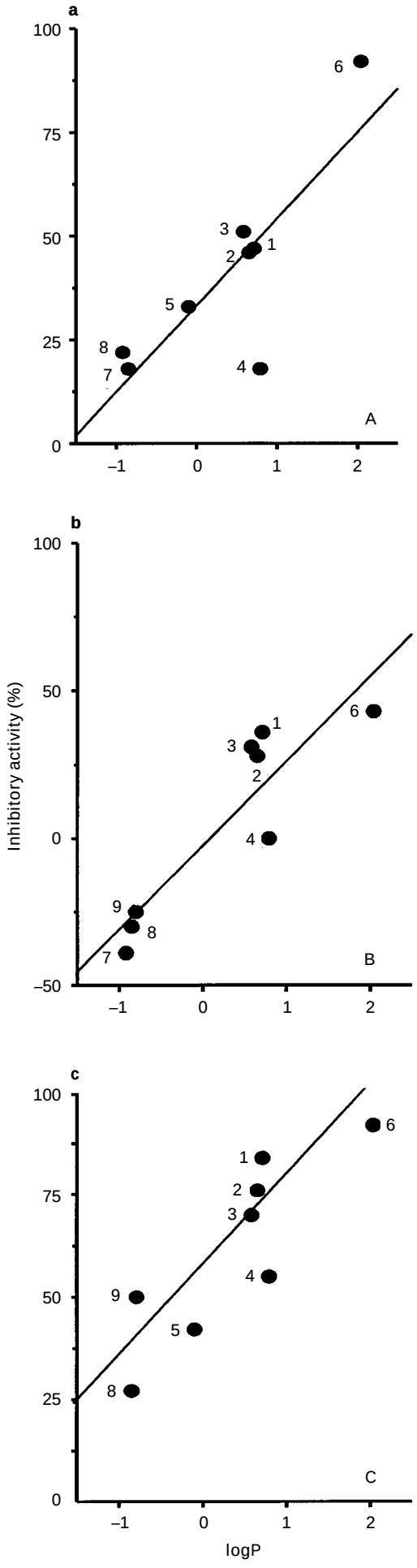
Discussion

In the present study interactions between MDR substrates/reversal agents and some classical organic cationic model compounds at the bile secretory level were studied in the intact liver of the rat. We used the rat isolated perfused liver technique since this model enables the study of drug secretion by hepatocytes in their normal architecture and polarity. The P-glycoprotein substrate doxorubicin and the organic cationic agents TBuMA and rocuronium were used as model compounds.

A profound inhibition of TBuMA secretion into bile was observed upon addition of vinblastine, but also TBuMA hepatic uptake was decreased by vinblastine. When calculating the liver/perfusate and bile/liver concentration ratios of TBuMA it was observed that the steep bile/liver ratios largely decreased upon addition of vinblastine, while concomitantly the liver to perfusate concentration ratio increased somewhat. This clearly indicated that inhibition of TBuMA transport occurred at the bile excretory level and that the decreased plasma disappearance after the addition of the inhibitors of TBuMA is the result of bile secretion inhibition rather than a direct inhibitory effect on the sinusoidal uptake.

The cumulative output of unchanged doxorubicin into bile reached a value of approximately 25% of the administered dose, which is comparable to earlier findings (Ballet *et al*., 1987). Both TBuMA and rocuronium are excreted into bile entirely as the parent compound (Neef *et al*., 1984; Shanks *et al*., 1993). The interactions at the secretory level, between MDR substrates/reversal agents and the cationic model compounds, were calculated from the cumulative biliary excretion, occurring after the administration of a potential inhibitor compared to controls (see Table 2). The excretion of TBuMA into bile was largely reduced by procainamide ethobromide (PAEB) at a 40 fold molar dose but hardly by a 4 fold molar dose. We also found a strong reduction in the PAEB biliary excretion upon addition of a 4 fold molar amount of TBuMA (data not shown). This indicates that mutual inhibition occurs during excretion of type 1 cations into bile, but that TBuMA probably has higher affinity for the transport system involved.

In the present study we observed that all of the MDR substrates/reversal agents studied strongly inhibited the biliary



excretion of TBuMA and to a lesser extent rocuronium (in the latter case with the exception of Dox). In contrast, the type 1 compounds TBuMA and PAEB did not significantly inhibit the rocuronium or doxorubicin biliary excretion rate, and also digoxin only slightly reduced the excretion of these compounds into bile. Doxorubicin excretion was strongly affected by the MDR substrates/reversal agents vinblastine, verapamil, quinine and quinidine, by the type 2 cation rocuronium and to a much lesser, albeit significant, extent by the type 1 compounds, TBuMA and PAEB. This may imply that all of these compounds share common export system(s). Yet, the relatively hydrophilic agents TBuMA and PAEB increased the cumulative biliary output of rocuronium. This may theoretically be explained by a trans-stimulant effect (accelerated exchange diffusion at the canalicular level), assuming the involvement of at least two different transport proteins of which one has bi-directional facilitated diffusion characteristics. The small type 1 organic cations may also displace rocuronium from intracellular organelles, since they have been shown to accumulate in lysozomes like rocuronium (Van Dyke *et al*., 1992; Moseley *et al*., 1993; Moseley & Van Dyke, 1995) and mitochondria (Saito *et al*., 1992; Röttele & Zimmermann, 1993; Steen *et al*., 1993), thereby increasing the availability of rocuronium for canalicular transport.

These differential inhibiting effects of the agents, in spite of their common basic (cationic) character, prompted us to elucidate whether other physico-chemical characteristics of the drugs studied are of importance in explaining the data observed. Therefore, we investigated the relative lipophilicity of the drugs studied and correlated the physicochemical parameter of the potential inhibitor with its inhibitory effect on the cumulative biliary output of the drugs tested. We found a significant correlation between the two parameters mentioned (Table 2). This suggests that the difference in hydrophobicity of the drugs investigated may be an important factor that determines the relative affinity of the studied drugs for the binding site(s) on P-glycoprotein or other transport protein(s). Alternatively, substrate recognition by transport proteins, at the canalicular level, may depend on the manner in which the particular substrate associates with the lipid bilayer in which the transport proteins are situated. In principle, relatively lipophilic cationic drugs may at least partly partition into the canalicular membrane and thereby become more available to integral membrane proteins involved in the substrate translocation process. The penetration of MDR substrates into the lipid moiety of the phospholipid bilayer seems to be a requirement for recognition by polyspecific drug transport proteins (Van Veen *et al*., 1996; Bolhuis *et al*., 1996). However, the molecular mechanism explaining the substrate recognition and the transmembrane translocation mechanisms of the broad range of substrates of MDR proteins remains to be elucidated.

The present study shows that the interaction between cationic drugs during transport at the bile canalicular level can be very profound and that the inhibitory activity seems to

Figure 5 The correlation between log P-value of potential inhibitors and their relative effects on the biliary excretion of doxorubicin (a), rocuronium (b) and tributylmethylammonium (TBuMA) (c). The inhibitory activity on the ordinate scale indicates the percentage of doxorubicin excreted into bile calculated from the time point at which an inhibitor is added, relative to amount excreted in the control in the same period. The numbers indicate the inhibition of doxorubicin excretion: (1) quinine, (2) quinidine, (3) verapamil, (4) digoxin, (5) rocuronium, (6) vinblastine, (7) tributylmethylammonium, (8) procainamidethobromide.

be related to the lipophilic nature of the compounds studied. The interactions of known P-glycoprotein substrates with some of the other classical organic cationic model drugs indicate that P-gp is a clear candidate for the secretion of various categories of cationic drugs into bile in the intact liver. Hydrophilic charged groups as well as hydrophobic portions in the molecule may determine the relative affinity for the various transport proteins including the P-gp isoforms. Competitive interactions with the transport proteins in the canalicular membrane are likely to play a role, in view of the fact that most of the interactions observed at the biliary excretion level were mutual.

We have recently obtained data with *mdr* gene 'knockout' mice that clearly support the idea that P-glycoprotein is involved: the biliary excretion of both type 1 and type 2 cationic compounds is at least 60% reduced compared with the controls (Smit *et al*., 1996). Since *mdr1a/1b* gene 'knockout' mice, that completely lack mdr1-type P-glycoprotein in liver, still have a residual excretion of organic cations into bile, we inferred that, besides P-gp, at least one other transport protein may be involved in cation excretion into bile. The multidrug resistance-related protein family (mrp) (Zaman *et al*., 1994; Loe *et al*., 1996), the phospholipid translocator mdr2 (Smit *et al*., 1994; Buschman & Gros, 1994) and the gene product of the sister of P-glycoprotein (Childs *et al*., 1995) as well as the proton:cation antiport system (Moseley *et al*., 1992a; 1996) are potential candidates. The impressive inhibition of doxorubicin excretion to less than 10% of the control values by vinblastine, may reflect an interaction with multiple carrier systems: it is unlikely that inhibition of mdr1a and mdr1b P-gp alone would lead to such a profound reduction of the biliary output.

Finally, the present data could imply that major interactions may occur during the hepatobiliary elimination of cationic drugs in patients. One example is the advocated combined use of antineoplastic drugs and of MDR reversal agents that, not only may improve the effective concentration of the cytostatic agents in multidrug resistant tumour cells, but also may lead to severe interactions at the hepatic-, intestinal and renal elimination levels or at the level of the blood brain barrier leading to toxic side effects (Schinkel *et al*., 1994; 1995; Mayer *et al*., 1996; Sparreboom et al., 1997).

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