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MDR2 P-glycoprotein-mediated lipid secretion and its relevance to biliary drug transport

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Abstract

Biliary excretion of xenobiotics is a complex process involving uptake into hepatocytes, internal sequestration and/or biotransformation, and transport into bile. Phospholipid secretion was previously supposed to be a passive process in which the phospholipid was released into bile by bile salt-induced extraction from the canalicular membrane by bile salts. This idea needed adaptation when it was recognized that phospholipid secretion not only depends on bile salt secretion but also requires the active participation of a protein, the mdr2 P-glycoprotein. In the current models mdr2 P-glycoprotein functions as a translocator of phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane. This translocation will result in alterations in composition and/or arrangement of membrane lipids in such a way that phospholipid can be extracted from the outer leaflet of the canalicular membrane by bile salts present in the canalicular lumen. Insight in the mechanisms of biliary phospholipid secretion could be useful for the development of strategies aimed at modifying the hepatic clearance of pharmaceuticals. Furthermore it could provide the necessary information to refine drug targeting protocols used for drugs that must exert their therapeutical effects in the biliary tree or intestinal tract.

Keywords: Flippase; Canalicular membrane; Protoporphyrin; Bile salt; Cyclosporin A; Organic anions; Membrane inserting drugs; Chlorpromazine; Amphipatic drugs; Lipid vesicles

Contents

1. Introduction	202
2. P-glycoproteins	202
2.1. P-glycoproteins can be functionally divided into two distinct classes.....	202
2.2. Tissue distribution of P-glycoprotein	203
2.3. The physiological function of mdr2 P-glycoprotein.....	203
2.4. The molecular mechanism of mdr2 P-glycoprotein	204
2.5. Mechanisms of P-glycoprotein mediated transmembrane transport.....	204
3. Hepatobiliary transport of lipids	205
3.1. Biliary lipids.....	205
3.2. Coupling of PC and cholesterol secretion	205
3.3. A modified mechanistic model of biliary lipid secretion	206
4. Implications of the new model of PL secretion for biliary drug transport	208
4.1. Functional aspects of lipid secretion	208
4.2. Secretion of compounds associated with biliary lipid	209
4.2.1. Protoporphyrin excretion as a model for lipid coupled hepatobiliary transport.....	209
4.2.2. mdr1 substrates and biliary lipids	209

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4.3. Drugs that influence lipid secretion.....	210
4.3.1. Uncoupling of PC and cholesterol excretion.....	210
4.3.2. Direct inhibition of lipid secretion.....	210
4.3.3. Membrane inserting drugs.....	211
5. Conclusions and perspectives.....	211
References.....	211

1. Introduction

Formation of bile by hepatocytes is a major, but not fully understood, function of the liver. It involves the vectorial transport of compounds such as bile salts, phospholipids, cholesterol as well as endo- and xenobiotics.

Hepatocytes are polarized epithelial cells with a basolateral (sinusoidal) domain that is in contact with blood plasma and an apical (canalicular) domain which is the site of primary bile formation. Transport of nutrients and metabolites from the blood to the hepatocyte occurs via distinct transport processes in the sinusoidal membrane.

Specific transporters, localized in the canalicular membrane, take care of the excretion of metabolites into an extracellular lumen. This extracellular lumen (bile canaliculus) is formed by the canalicular membranes of neighbouring hepatocytes, and forms a three dimensional network of channels that anastomose into bile ductules and the common bile duct.

The net result of the apical and sinusoidal localized transport processes determine the composition of primary bile. Besides the excretory function of the hepatocyte for metabolic waste products there is also secretion of bile salts which assist in fat dispersion and absorption in the intestinal tract. In addition, the canalicular membrane provides a site for extensive lipid secretion into bile. This is critical for cholesterol homeostasis and efficient absorption of dietary lipids in the intestine, while it also mitigates the detergent action of the high bile salt concentrations in the biliary tree.

The secretion of bile salts and organic anions provides an osmotic gradient between canalicular lumen and plasma, which causes a water flow through the tight junctions sealing the adjacent hepatocytes. The secretion of bile salts and organic anions into bile is a major factor determining hepatic bile flow.

Insights in the mechanism of hepatobiliary lipid secretion could possibly also be beneficial for the

understanding of biliary clearance of similar compounds or compounds of which the secretion is strongly coupled to the excretion path of the lipid phase. In addition, proper understanding of hepatic transport may lead to the development of compounds that can be specifically targeted to various cell types in the liver and intestinal tract.

2. P-glycoproteins

It is known already for a long time that mammalian cells can develop a resistance against a variety of functionally and structurally unrelated drugs upon selection with a single cytotoxic drug. This phenomenon of acquired resistance is known as multidrug resistance (MDR). In 1976 Juliano and Ling [1] discovered a plasma membrane protein that is overexpressed in colchicine-resistant tumor cells. They called it P-glycoprotein (Pgp) because it was assumed to be involved in the permeation of these drugs across the plasma membrane. It is now clear that one of the major mechanisms of MDR is the overexpression of P-glycoproteins (170 kD) which actively pump these compounds out of the cell, maintaining a low intracellular concentration and thereby protect the cell against toxicity (reviewed in [2,3]).

2.1. P-glycoproteins can be functionally divided into two distinct classes

The cloning of various P-glycoproteins revealed a small group of isozymes encoded by highly homologous genes. In mice three genes were found to encode for Pgp's, *mdr1*, *mdr2* and *mdr3*. Because of their high homology, *mdr1* and *mdr3* were later renamed *mdr1b* and *mdr1a*, respectively [4]. Two genes were characterized in humans, *MDR1* and *MDR3*. High homology was observed between the mouse *mdr2* and the human *MDR3* genes. These Pgp encoding genes are members of the superfamily

of ABC transporters, a large family of proteins which possess ATP-binding domains and are involved in active transmembrane transport [2,3,5].

Pgp's show a very high degree of inter- and intra-species homology [2], nevertheless there is a striking functional difference between the individual *mdr* genes. Transfection of the human *MDR1* or mouse *mdr1a* or *mdr1b* in cell lines reveals that these genes can confer multidrug resistance against amphipathic drugs, while *MDR3* and *mdr2* cannot (reviewed in [6]).

2.2. Tissue distribution of P-glycoprotein

The apparent functional difference between the two classes of P-glycoproteins seems to be reflected by the difference in expression patterns in normal tissue. The human *MDR1* is distributed throughout the body. Immunohistochemical and RNase protection assays show *MDR1* Pgp expression in the proximal tubuli of the kidney, tissues in the gastrointestinal tract, bile canaliculi and bile ductules, the capillary endothelial cells of testis and brain, endometrium of the uterus, and adrenal gland [7–15].

The murine counterparts of *MDR1* Pgp, *mdr1a* and *mdr1b*, match the expression of the human gene although they seem to have a complementary and partially overlapping distribution. The non-MDR conferring mouse *mdr2* Pgp and human *MDR3* Pgp are predominantly expressed in the hepatocyte and to a lower extent also in muscle, and spleen [16–18].

The high expression of *mdr2/MDR3* Pgp in the hepatocyte is exclusively restricted to the canalicular membrane [8,18] suggesting a role in hepatobiliary transport.

2.3. The physiological function of *mdr2* P-glycoprotein

The localization of *MDR1/mdr1a-mdr1b* P-glycoproteins and their ability to transport naturally occurring cytotoxic compounds across membranes in vitro, suggests a physiological role in the protective mechanism against toxic insults or transport of endogenous substrates [19–24].

The pursuit of the physiological function of *mdr2* P-glycoprotein finally succeeded in 1993 when knockout mice for the *mdr2* gene were produced [25]. The phenotypical characteristics of *mdr2*

knockout mice almost directly reflected its putative physiological function. Bile formation is severely affected in these animals. The most prominent change in bile composition represents a virtual absence of phospholipid and a dramatic decrease in cholesterol, while bile salt secretion is normal. Glutathione secretion is severely decreased as well. Analysis of bile of mice heterozygous for *mdr2* gene showed a normal composition except a 40% decrease in the phospholipid content. This strongly suggested that *mdr2* Pgp is primarily involved in the biliary secretion of phospholipids. Based on its homology with the other P-glycoproteins it was hypothesized that *mdr2* Pgp is a transporter as well and thus, that it might function as a flippase which translocates phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane [25].

Because the *mdr1a* Pgp is also expressed in the canalicular membrane it was interesting to evaluate its physiological function in hepatobiliary transport. Once again generation of genetically modified animals provided some straightforward answers. The phenotype of *mdr1a* knockout mice was entirely different from the *mdr2* knockout. Normal bile secretion parameters in *mdr1a* knockout mice were not affected (see Table 1) and no liver pathology was found. These animals showed accumulation of amphipathic drugs like ivermectine and vinblastine in brain tissue, which suggests that *mdr1a* Pgp plays an important role in extrusion of these compounds across the blood brain barrier [26]. The role of this

Table 1
Bile secretion in control mice and in mice with a homozygously disrupted *mdr1a* or *mdr2* gene

Mouse genotype	(+/+)	<i>mdr1a</i> (–/–)	<i>mdr2</i> (–/–)
Bile flow ($\mu\text{l}/\text{min} \cdot 100 \text{ g}$)	9.8 $\pm$ 1.5	7.3 $\pm$ 1.4	16.9 $\pm$ 3.3 ^a
Bile salt secretion (nmol/min $\cdot$ 100 g)	309 $\pm$ 88	359 $\pm$ 139	394 $\pm$ 88
Phospholipid secretion (nmol/min $\cdot$ 100 g)	26.8 $\pm$ 4.5	30.0 $\pm$ 9.0	0.3 $\pm$ 0.5 ^a
Cholesterol secretion (nmol/min $\cdot$ 100 g)	2.7 $\pm$ 0.7	2.5 $\pm$ 0.4	0.08 $\pm$ 0.07 ^a

Bile flow and bile components were determined in bile samples that were obtained during the first 10 min directly after cannulation of the gallbladder. Data represent means $\pm$ S.D. of 7 animals from the (+/+) and the *mdr2* (–/–) strain and from 3 animals from the *mdr1a* (–/–) strain. Significant differences were determined by Student's *t*-test. ^a *p* < 0.001.

Pgp in canalicular secretion of amphipathic drugs could not be elucidated because in the *mdr1a* knockout increased expression of the *mdr1b* gene was observed. In order to circumvent this problem, mice with the combined disruption of both *mdr1a* and *mdr1b* genes were produced. Characterization of the phenotypes of these double knockouts is underway.

2.4. The molecular mechanism of *mdr2* P-glycoprotein

The hypothesis of *mdr2* Pgp being a phospholipid flippase was recently supported by experiments of Ruetz and Gros [27] who transfected the *mdr2* gene in a yeast secretion mutant. Using this experimental model the translocation of NBD-labeled phosphatidylcholine (NBD-PC) from the outer to the inner leaflet of the secretory vesicles from these yeast cells was determined. ATP-dependent translocation of NBD-PC was observed which was specific for *mdr2* Pgp. Vesicles from yeast cells that were transfected with *mdr1* or with the transfection vector alone were not able to transport the substrate in a ATP-dependent fashion. A small fraction of PC-molecules was translocated in this assay. This may, however, be expected since the translocation process induces a phospholipid imbalance between the inner and outer leaflet, which is thermodynamically highly unfavorable. Thus, in the absence of net extraction of phospholipid from the trans-side of the bilayer, the translocation will halt. Unfortunately, little information was obtained on the substrate specificity of transport. It was not described whether the NBD-moiety was present in the head group or in the fatty acid tail of phosphatidylcholine. Unexpected was the inhibition of translocation by low concentrations of verapamil, which is an inhibitor of *mdr1* Pgp. This suggests that verapamil is able to inhibit *mdr1* as well as *mdr2* Pgp.

Further evidence supporting the flippase function of *mdr2* Pgp was provided by Smith et al. [28] who used fibroblasts from transgenic mice that express *MDR3*, the human homologue of *mdr2* Pgp. After metabolic labeling of intracellular phosphatidylcholine with radioactive choline, translocation from the inner to the outer leaflet was assayed and this was compared with normal mouse fibroblasts which do not express *mdr2*. Translocation of radioactive phos-

phatidylcholine to the outer leaflet was measured by the possibility to exchange with phosphatidylcholine-transfer protein and liposomes in the medium. In *MDR3* expressing fibroblasts a more rapid translocation of PC was observed than in control fibroblasts. Both studies provided suggestive evidence in favor of the translocator model in which *mdr2* Pgp is an ATP-dependent phosphatidylcholine translocator.

2.5. Mechanisms of P-glycoprotein mediated transmembrane transport

Data derived from many studies (reviewed in [2,3,29]) were used to deduce a speculative model for Pgp structure. P-glycoproteins are glycosylated plasma membrane proteins (140–170 kDa) of about 1280 amino acids long. They consist of two duplicated halves each containing six putative membrane spanning regions with a highly conserved ATP-binding domain.

To date this structure model alone is not sufficient to elucidate the mechanism of drug transport. It remains to be established whether the P-glycoprotein really functions as a conventional transporter or, alternatively, acts as a membrane extruder that merely expels amphiphilic compounds entering the membrane by non-ionic lipid diffusion [30]. The latter concept has been called the 'hydrophobic vacuum cleaner' model and may bear resemblance with phospholipid flippase-type of translocation as suggested by Smith et al. [25]. This concept implies that access of substrate to the transport protein occurs directly from the lipid phase and that the drug is flipped from the inner leaflet of the bilayer to the outer. This model also predicts that the P-glycoproteins could decrease the initial uptake rate of the substrate in cells in vitro since it would not differentiate between drug entering the membrane from the inner or outer cellular space [13]. Valverde et al. [31] and Gill et al. [32] reported an increase in cell swelling-activated Cl^- currents in several cell-types expressing P-glycoprotein. Gottesman and Pastan [3] have tried to combine the different reported transport functions of Pgp for protons, chloride and amphipathic compounds in an alternative model. In this hypothetical model ATP hydrolysis is linked to the transport of protons into the transporter, with chloride following passively. A water flow is generated by the osmotic gradient resulting from the ions inside

the lumen of the transporter. Consequently membrane localized amphipathic drugs entering the water phase are excreted.

Both models could explain the broad specificity for amphipathic compounds but recent data shed doubt to the latter model. These studies have shown that the chloride transporting feature is not a feature of P-glycoprotein itself but that Pgp expression regulates an endogenous channel [5,33,34].

3. Hepatobiliary transport of lipids

Cholesterol and phospholipids are, in quantitative terms, major constituents of the organic fraction of bile.

The secretion rate of cholesterol into human bile is 0.8–1.2 g per day and into rat bile it amounts up to 15 mg per day. The PL secretion rates are 3–4 g per day and 200 mg per day, respectively [35,36]. The 'classical' model for biliary lipid secretion supposed the passive extraction of canalicular membrane lipids by micellar bile salts in the canalicular lumen. Recently, it was demonstrated that *mdr2* P-glycoprotein expressed in the liver also has a major regulatory function in lipid secretion from the hepatocyte to the bile. Both factors seem to be important for efficient lipid secretion and can be fitted in a new hypothetical secretion model.

3.1. Biliary lipids

In all animal species studied, bile phospholipids almost exclusively consist of phosphatidylcholine (PC) molecules that have a specific, relatively hydrophilic, fatty acid composition. The *sn*₁ position of biliary PC usually is occupied by the saturated fatty acid species palmitate (16:0) whereas the *sn*₂ position invariably contains an unsaturated species, predominantly oleate (18:1) or linoleate (18:2). The fatty acid composition of biliary PC contrasts with that of PC in the canalicular membrane which has a high content of arachidonate (20:4) in the *sn*₂ position [37–39]. The major constituents of hepatic membranes, sphingomyelin and phosphatidylethanolamine, are present in bile only in small amounts under normal conditions. Apparently hepatocytes selectively recruit specific PC species for secretion into bile and/or effectively exclude other phos-

pholipids from entering this pathway. Cholesterol is present in bile exclusively in its unesterified form. The lipids in bile are present in different aggregated states. Shape, size and composition of these aggregates is determined by the concentration of these lipids relative to that of the biliary bile salts as well as by their physicochemical characteristics.

A large body of evidence is available to indicate that simple micelles, consisting of bile salts and cholesterol, mixed micelles, containing bile salts, cholesterol and phospholipids, and unilamellar vesicles, consisting of cholesterol and phospholipids and trace amounts of bile salts, can co-exist as biliary lipid carriers in a dynamic, i.e. interchangeable, fashion [40–42].

Multilamellar vesicles, liquid cholesterol crystals, can be present in bile supersaturated with cholesterol [40]. Supersaturation of bile is a prerequisite for the formation of cholesterol gallstones.

3.2. Coupling of PC and cholesterol secretion

Data from many studies suggest that the secretion of phospholipid and cholesterol occurs in a coupled fashion. Experimentally induced alterations in biliary phospholipid secretion result in a similar alteration in cholesterol secretion [43]. It has been recognized however that under certain metabolic conditions deviations can occur [44–47]. It is also evident that the secretion of cholesterol and phospholipids is, at least in part, governed by bile salts present in the canalicular lumen.

The mode of action by which bile salts regulate lipid secretion is still a matter of debate. In most species the relationship between bile salt secretion and that of cholesterol and phospholipids appears to be curvilinear. In addition, many studies have demonstrated a direct positive relation between bile salt hydrophobicity and the amount of lipid that can be secreted [37,48–52]. The relative hydrophobicity of a bile salt is reflected in its critical micellar concentration (CMC). The observed quantitative differences between the amount of lipid secreted per amount of bile salt in different species could be explained by differences in bile salt species present in the bile salt pool [53].

Intracanalicular bile salt micelle formation cannot be the only factor regulating biliary lipid secretion. This would not be in agreement with a number of

observations: (1) Presence of cholesterol–phospholipid vesicles in native bile samples [54–56]; (2) the isolated hypo- or hypersecretion of phospholipids [46] and of cholesterol [57,58] under certain experimental conditions; (3) the highly species-specific lipid-to-bile salt ratio [53].

Recent work indicates the presence of (an) additional regulatory mechanism(s) involving canalicular membrane proteins, specifically the *mdr2* P-glycoprotein [25]. In a recent paper, Ruetz and Gros, studied the cooperative relationship between *mdr2* Pgp expression and bile salt in the process of biliary lipid secretion [59]. Using the same heterologues *mdr2* Pgp expression system in yeast *sec6-4* mutants as mentioned in Section 2.4, they showed that secretion of PC transformation in secretory vesicles transfected with *mdr2* Pgp is significantly stimulated by the presence of taurocholate in these vesicles. The non-micelle forming bile salt taurodehydrocholate does not cause such a stimulation.

3.3. A modified mechanistic model of biliary lipid secretion

The mechanism of the *mdr2* P-glycoprotein action remains speculative at the moment. Recently published work on phospholipid translocation in rat liver canalicular membranes by Berr et al. [60] as well as the proposed mode of action of P-glycoproteins [30], put forward an attractive model, involving the action of the protein as a ‘flippase’, i.e. translocating bile-specific PC from the inner to the outer leaflet of the membrane.

For many years the most commonly accepted model for biliary phospholipid secretion included passive extraction of canalicular membrane lipids by micellar bile salts in the canalicular lumen (for reviews see [37,51]). PC was thought to be transported to the canalicular membrane via PC-rich vesicles that are derived from the endoplasmic reticulum where the lipid is synthesized. After insertion in the membrane, PC was thought to reside in microdomains that were extracted from the membrane by the canalicular bile salts. Although never demonstrated in canalicular membranes up to now, such microdomains have been observed in other, in vitro systems [61].

The action of bile salts was thought to consist of

an interaction of bile salt monomers or micelles with the microdomains leading to budding of the microdomain bilayer and subsequent pinching off of PC-rich vesicles. The presence of phospholipid vesicles in bile was observed, although their emanation from the canalicular membrane is hard to prove [55,56]. Bile salts have been shown to induce the pinching-off of ‘bile-type’ membrane lipids from red blood cell membranes in the form of vesicles [62,63].

Biliary phospholipids consist almost exclusively of phosphatidylcholine (PC; >95%) whereas the canalicular membrane also contains sphingomyelin (SM; $\pm 20\%$), phosphatidylethanolamine (PE; $\pm 20\%$) and phosphatidylserine (PS $\pm 20\%$) [39,64]. The presence in the membrane of sphingomyelin, cholesterol and PC makes it more rigid than the proposed microdomains, that are particularly rich in PC with shorter acyl chains. As a consequence, extraction of PC from these fluid microdomains by luminal bile salt micelles was thought to proceed much easier than from other parts of the membrane.

The classical concept of bile salt-induced secretion of PL and cholesterol through budding of the membrane involves both leaflets of the membrane. Inclusion of flippase action of *mdr2* Pgp in this model has no functional relevance because it could not explain the absence of PL secretion in the absence of *mdr2* Pgp. Therefore a new model was generated that recognized the involvement of asymmetry of the canalicular membrane bilayer.

The first step in this model is the supply of PC to the canalicular membrane. The intracellular trafficking of PC in hepatocytes has been reviewed by Coleman and Rahman [37] and Oude Elferink et al. [65] and concerns probably two main routes; transport mediated by specialized PC transfer proteins and/or intracellular vesicles. If PC is supplied via phosphatidylcholine transfer protein it will be delivered to the inner leaflet and *mdr2* Pgp then translocates PC from the inner to the outer leaflet of the membrane. If, however, vesicular supply is the major mechanism, a substantial amount of PC molecules will arrive directly in the outer leaflet of the membrane because PC will be present in both leaflets of such a vesicle. In that case a substantial amount of PC is already present in the outer leaflet of the canalicular membrane and therefore available for bile salt-mediated secretion without the need for a flippase. The absence of PL in bile from *mdr2* (–/–)

suggests that at least in these animals the vesicular route is not important.

The mechanism by which bile salts induce lipid secretion has not yet been elucidated. Recent data from Crawford et al. provides suggestive evidence for bile salt induced vesiculation [66]. Using an ultra-rapid fixation technique they were able to demonstrate bile salt dependent formation of vesicles on the exoplasmatic leaflet of the canalicular membrane. Preliminary results demonstrated a greatly reduced number of vesicles in the *mdr2* knockout mice and 50% reduction in the heterozygotes [67]. The role of *mdr2* Pgp in this process could be the induction of instable microdomains in the outer leaflet of the canalicular membrane through the production of local excess of PL. Under the influence of bile salts, these instable structures ultimately shed from the membrane in a vesicular form, since it has

been observed that lipids in primary bile exist as vesicles [55]. Whether these vesicles indeed represent intermediate structures formed during the process of biliary lipid secretion needs to be proven.

The above described new hypothetical model of biliary lipid secretion is depicted in Fig. 1. The left panel represents the situation in the absence of *mdr2* P-gp mediated PC translocation. Canalicular BS have leached PL from the outer leaflet. This has induced an increase in outer leaflet cholesterol and sphingomyelin which is known to make the canalicular membrane highly resistant to the detergent action of bile salts. This is in agreement with the observation that infusion of the liver of *mdr2* (–/–) knockout mice with bile salts that are more hydrophobic than the ones present in their endogenous pool, still resulted in an almost negligible secretion of phospholipids. The panel in the middle represents a

Fig. 1. Hypothetical model of the mechanism of *mdr2* Pgp-mediated lipid secretion. Phosphatidylcholine is supplied to the membrane via phosphatidylcholine-transfer protein which inserts PC into the inner leaflet. *mdr2* Pgp translocates PC to the outer leaflet into PC-rich microdomains and this ATP-dependent process leads to phospholipid imbalance in the membrane. Luminal bile salt micelles or monomers further destabilize these domains and this leads (via an unknown mechanism) to the formation and release of vesicular structures. In the absence of PC-rich domains, luminal bile salt micelles are unable to extract phospholipids from the (outer leaflet of the) membrane. This may be caused by a high proportion of glycosphingolipids, sphingomyelin and cholesterol in the outer leaflet. Luminal bile salts can, however, extract cholesterol directly from the outer leaflet. This extraction is more efficient when mixed micelles of bile salts and PC are present because these have a higher affinity for cholesterol.

situation in which the *mdr2* Pgp is active but there is no bile salt present in the canalicular lumen to extract the PL-rich regions from the membrane. The PL secretion in the normal state is depicted in the panel on the right. Both bile salt and *mdr2* Pgp are present and can maintain the desired PL secretion into the canalicular lumen in a cooperative fashion.

Cholesterol molecules in the outer leaflet of the membrane could laterally diffuse into these microdomains and than be secreted together with the phospholipid. In this model, a close relation between cholesterol and phospholipid secretion rates is expected which will be determined by the cholesterol content of the canalicular membrane. This model is supported by the observation in many studies that cholesterol and phospholipid secretion are tightly coupled. It does, however, not explain the substantial cholesterol secretion that was observed in *mdr2* knockout mice in the virtual absence of phospholipid secretion. An alternative mechanism could be that bile salt micelles directly extract cholesterol from the outer leaflet of the membrane. The efficiency of this extraction is determined by the cholesterol solubilizing capacity of bile salts and the presence of phospholipids further increases this efficiency because mixed micelles of bile salts and phospholipids have a higher cholesterol solubilizing capacity [68]. In the latter model cholesterol secretion would thus only be secondarily dependent on phospholipid secretion.

4. Implications of the new model of PL secretion for biliary drug transport

The recognition of the role of *mdr2* Pgp in biliary phospholipid secretion could be a very important step towards the unraveling of the physiological mechanism of lipid secretion. In the future, this mechanism then possibly can be used to control PL and cholesterol secretion and/or the delivery of (designer) drugs to the biliary tree or the intestinal tract.

At present, comprehension of the mechanism of biliary PL secretion is important to have proper understanding of the various (putative) functional aspects of biliary lipid secretion.

4.1. Functional aspects of lipid secretion

Conversion of cholesterol into bile salts is the major degradation pathway for the lipid. Bile salts are almost exclusively secreted into bile. Thus, the biliary pathway represents the major route for the removal of cholesterol, either as such or in the form of bile salt, from the body and functions as a crucial factor in the maintenance of cholesterol homeostasis [69]. The highly efficient reabsorption of bile salts from the intestine appears to be somewhat contradictory to the homeostatic function of biliary cholesterol secretion. However, the controlling step in cholesterol metabolism could be mainly located in the intestine and to less extent in the liver.

Biliary phospholipids play an important role in the absorption of dietary lipids from the intestine, in addition to the well-established function of bile salts in this process. Studies by Davidson et al. [70] have provided evidence for a physiologic role of biliary phospholipids in the regulation of intestinal apolipoprotein B48 expression, an apolipoprotein essential for proper assembly and secretion of chylomicrons containing the absorbed dietary fats.

In bile, the presence of cholesterol and phospholipids may protect the hepatocytes and the cell lining of the bile ductules from the cytotoxic effects of the (detergent) bile salts. This function is probably best exemplified by the development of liver disease in the *mdr2* P-glycoprotein knock-out mice produced by Smit et al. [25]. The liver pathology of these mice is mild directly after birth but progresses to severe liver disease during lifetime and ends with the development of hepatocellular carcinoma. Liver histology showed degenerative features (of hepatocytes) throughout the lobule, irregular size with nuclear polymorphism, and focal necrosis with formation of eosinophilic bodies. In these mice the bile ducts were affected as well, with extensive portal expansion owing to ductular proliferation. This pathology may be the result of prolonged exposure of apical membranes along the biliary tree to the detergent action of lipid-free bile salt micelles [71].

Finally the biliary lipid could serve as carriers for lipophilic compounds in their routing from hepatocyte to the intestine. In the following sections some experimental data connected to this putative carrier function will be discussed.

In addition we will discuss the possible role several drugs play in manipulating this transport function by interfering in the process of biliary lipid secretion.

4.2. Secretion of compounds associated with biliary lipid

Based on both in vitro and in vivo studies, it has been proposed that lipid vesicles may act as transport carriers for hydrophobic anions such as indocyanine green (ICG) and protoporphyrin (PP). As shown by Tazuma et al. [72,73] and Berenson et al. [74], these hydrophobic organic anions show a high affinity for the (vesicular) lipid fraction in bile and it has been suggested that vesicular structures act as carriers of these compounds during transit from the liver to the intestine. Although this is a new concept, structurally and/or secretion characteristics of several compounds make them candidate compounds for such an excretion pathway.

Some data is available concerning biliary protoporphyrin excretion. The hypothetical mechanism that can be derived from these data is discussed in more detail in the next sub-section and may be of importance for other compounds as well.

4.2.1. Protoporphyrin excretion as a model for lipid coupled hepatobiliary transport

Protoporphyrins are large planar hydrophobic molecules that are virtually insoluble in water at physiological pH. Protoporphyrin are intermediates in heme synthesis and excreted from the body solely by secretion into bile and subsequent elimination via faeces. It is known that biliary protoporphyrin excretion in rat liver is greatly increased by infusion of bile salts and that protoporphyrin secretion shows a curvilinear relationship with bile salt secretion. Protoporphyrin secretion reaches a plateau phase at high bile salt secretion rates and taurocholate (TC) is a more potent inducer of protoporphyrin secretion than the more hydrophilic ursodeoxycholate [75–77].

Infusion of taurocholate induces a 150-fold increase in protoporphyrin excretion compared to controls. Berenson et al. [78] studied the relation between canalicular bile salts and protoporphyrin excretion in the perfused rat liver. These elegant studies revealed that bile acids facilitate the biliary

translocation of protoporphyrins in the same manner as it effects biliary phospholipid secretion. Injection of 5 μmol TC together with 4 μmol of protoporphyrin to the portal venous catheter resulted in a peak in biliary porphyrin secretion and this peak coincided with the peak of PL secretion. Injection of TC 12 minutes after injection of protoporphyrin caused an equal delay in PL and protoporphyrin secretion.

Because of their strong relation, it was speculated that *mdr2* Pgp may both influence PL and protoporphyrin secretion by a similar mechanism. This possibility was further investigated by Beukeveld et al. [79]. They determined the secretion of protoporphyrin in *mdr2* knockout mice and controls. Biliary secretion of endogenous protoporphyrin was strongly reduced (90%) in knockout mice compared to the controls.

The precursor molecules of protoporphyrin, coproporphyrin isomers I and III, showed no significant reduced secretion in the *mdr2* Pgp knockout mice compared to controls. The mechanism of protoporphyrin excretion seems to be different from the coproporphyrin isomers. In contrast to protoporphyrin, the coproporphyrin isomers can also be excreted via the urine and they seem to be excreted into bile by the canalicular multispecific organic anion transporter while protoporphyrin cannot be excreted via this mechanism [79].

Whether protoporphyrin is transported directly by *mdr2* Pgp or is translocated through the membrane by another mechanism is not known. Secretion of protoporphyrin could be explained by two mechanisms: (1) vesicles present in the canaliculus could accept protoporphyrin, residing in the canalicular membrane or (2) protoporphyrin and (vesicular) lipids could simultaneously be discharged from the outer leaflet. Speculating on these hypothetical mechanisms, a new group of related compounds emerge, the excretion of which may be also associated with the carrier function of biliary lipids.

4.2.2. *mdr1* substrates and biliary lipids

An important group of compounds that might use lipid vesicles as carriers to the intestine are the substrates of *mdr1* P-glycoprotein [19–24]. These amphipathic substrates could enter the membrane by diffusion. As discussed earlier in Section 2.5, the

molecular mechanism of Pgp-mediated secretion remains to be established but it is likely that *mdr1* Pgp, in analogy with *mdr2* Pgp, translocates substrates from the cytosolic site of the cell, or from the inner leaflet to the outer leaflet of the plasma membrane. At the canalicular level translocating the substrates to the outer membrane leaflet would be sufficient when the substrates are removed from this part of the membrane by other mechanisms. The combined action of *mdr2* Pgp and bile salts could provide a way to dispose the compound by including it into the lipid phase to be expelled from the membrane. Capture of the compound in a lipid environment also impedes the substrate to reenter the plasma membrane by diffusion.

The transport of hydrophobic compounds through the biliary tract in lipid vesicles could be a general used mechanism, also for compounds that are transported over the canalicular membrane in a non-lipid associated fashion and enter the lipid vesicles after disposition in the canalicular lumen.

4.3. *Drugs that influence lipid secretion*

The proposed carrier function of biliary lipids could, in the future, possibly be used to develop new strategies for drug targeting or delivery or to control the secretion of compounds that are eliminated from the body by the liver. Several drugs are known to be able to interfere with canalicular lipid secretion. The mode of action is not always identical and the next sections will shortly address their possible mechanisms.

4.3.1. *Uncoupling of PC and cholesterol excretion*

It is well known that several hydrophilic organic anions can inhibit biliary lipid secretion without affecting bile salt secretion [80–82], a process referred to as ‘uncoupling’. This phenomenon has been used experimentally to determine the site of action of bile salt induced lipid secretion. Evidence has been provided that, under physiological conditions, bile salt induced lipid secretion is regulated at the level of the canalculus and within the hepatocyte. In these studies, reviewed by Verkade et al. [47], it was first shown that hydrophilic organic anions, that predominantly interact with biliary bile salts, are able to uncouple lipid from bile salt secretion in normal but not in mutant (GY/TR⁻) rats

[83,84]. These rats lack canalicular ATP-dependent organic anion transport and therefore show impaired bile secretion and increased intracellular concentrations of the uncoupling agents [85,86]. This observation demonstrates that uncoupling takes place within the canalculus and strongly suggests that the interaction of organic anions with bile salts is exerted after secretion of the bile salts across the bile canalicular membrane [85,86].

The uncoupling of PL and cholesterol from bile salt secretion has been reported as a side effect for therapeutical use of organic anions. Patients receiving ioglycamide, a compound used as contrast medium for radiographic inspection of the biliary tree, showed a depression of phospholipid and cholesterol secretion while bile salt secretion remains normal [87].

This anion is similar to iodipamide, which in addition to uncoupling activity in rats [88] can cause hepatotoxicity [89]. Theoretically, liver damage could occur by reducing the lipid to bile salt ratio in the canalicular lumen. The biliary lipids are less able to counteract the detergent action of the canalicular bile salts, that can become toxic to membranes lining the lumen of the biliary tract. Although this pathophysiological mechanism is speculative, iodopamide has been shown to induce liver damage in rats injected with iodopamide. Electronmicroscopic analysis of the liver revealed accumulation of lipid droplets 17 days after the injection of iodopamide [90]. Thus, the uncoupling effect of certain cholephilic anions calls for care in the chronic use of these compounds.

4.3.2. *Direct inhibition of lipid secretion*

Direct inhibition of biliary lipid secretion can be achieved by interfering with the driving force(s) of this process; BS secretion and/or lipid translocation by the *mdr2* Pgp. As mentioned before, bile flow is critically dependent on transport of bile salts and organic anions into the canalicular lumen because it is an osmotically driven process. Restriction of bile salt secretion usually leads to reduced bile flow and eventually to cholestasis. Several cholestatic drug and agents have been identified (reviewed in [65]). One of them is the immunosuppressive drug cyclosporin. Cyclosporin A interacts with many aspects of the bile salt secretory pathway. Firstly it inhibits Na⁺- dependent taurocholate uptake in sinusoidal

membrane vesicles and cultured hepatocytes [91,92]. Secondly it is an inhibitor of bile salt synthesis [93] and finally it inhibits canalicular located ATP-dependent secretion of bile salts. Since the canalicular bile salt transporter most likely controls bile salt secretion, inhibition of this step is probably the primary cause of the cholestasis induced by cyclosporin [94,95].

Cyclosporin A also turns out to be a very potent inhibitor of *mdr1* Pgp and other ATP-dependent translocators [96] and it could be an inhibitor for *mdr2* Pgp as well. This brings us to the inhibition of the second factor involved in biliary lipid secretion, the *mdr2* Pgp. Ruetz and Gross showed a reduced ATP-dependent translocation of NBD-PC (described in Section 2.4) when verapamil was present. Verapamil is a well established inhibitor of P-glycoprotein-mediated drug transport like cyclosporin A.

4.3.3. Membrane inserting drugs

Another way to interfere with membrane transport capability is alteration of the plasma membrane fluidity. The anti-psychotic and anti-emetic drug chlorpromazine is a well known example of compounds that inhibit membrane transport in such a way. Chlorpromazine inserts preferentially into the cytoplasmic half of the plasma membrane, as has been shown for the erythrocyte plasma membrane. This intercalation causes a expansion of the inner leaflet of the plasma membrane relative to the outer leaflet which causes a invaginated or cupped form of the membrane. Sheetz and Singer [97] described the alteration of rat liver plasma membrane fluidity and the concomitant diminished ATP-ase activity by chlorpromazine [98,99]. Samuels and Carey described the effects of chlorpromazine hydrochloride on Mg^{2+} - and Na^{+} -, K^{+} -ATPase activity of canalicular-enriched liver plasma membranes. Infusion of tauroursodeoxycholate into the isolated perfused rat liver has been shown to reverse the cholestatic effect of chlorpromazine together with an increased excretion of chlorpromazine excretion into bile. It was speculated that removal of chlorpromazine from the membrane and correction of the membrane fluidity resulted in restoring the ATP-ase activities leading to normal bile formation. The liver pathology induced by chlorpromazine in humans has been linked to the development of vanishing bile duct syndrome that eventually leads to biliary cir-

rhosis [100]. This is very interesting because in *mdr2* knockout mice, as described earlier, the bile ducts were affected as well.

5. Conclusions and perspectives

New insights in the mechanism of hepatobiliary lipid secretion does not directly give access to new clinical applications of this concept. However it can provide us the tools to hypothesize and explore new aspects concerning the role of bile formation in the maintenance or reestablishment of health in the human or animal body.

The concept of biliary lipids functioning as carriers for hydrophobic compounds is interesting and control of the lipid secretion could be a new approach in the development of methods to capture certain drugs in the hepatocytes, to deliver them to the biliary tree or to increase their elimination from the body.

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