Silymarin as a New Hepatoprotective Agent in Experimental Cholestasis: New Possibilities for an Ancient Medication

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Abstract: Silymarin is a purified extract from milk thistle (*Silybum marianun* (L.) Gaertn), composed of a mixture of four isomeric flavonolignans: silibinin (its main, active component), isosilibinin, silydianin and silychristin. This extract has been empirically used as a remedy for almost 2000 years, and remains being used as a medicine for many types of acute and chronic liver diseases. Despite its routinely clinical use as hepatoprotectant, the mechanisms underlying its beneficial effects remain largely unknown. This review addresses in detail a number of recent studies showing a novel feature of silymarin as a hepatoprotective drug, namely: its anticholestatic properties in experimental models of hepatocellular cholestasis with clinical correlate. For this purpose, this review will cover the following aspects:

1. The chemistry of silymarin, including chemical composition and properties.
2. The current clinical applications of silymarin as a hepatoprotective agent, including the mechanisms by which silymarin is thought to exert its hepatoprotective properties, when known.
3. The physiological events involved in bile formation, and the mechanisms of hepatocellular cholestasis, focusing on cellular targets and mechanisms of action of drugs used to reproduce experimentally cholestatic diseases of clinical interest, in particular estrogens and monohydroxylated bile salts, where anticholestatic properties of silymarin have been tested so far.
4. The recent findings describing the impact of silymarin on normal bile secretion and its novel, anticholestatic properties in experimental models of cholestasis, with particular emphasis on the cellular/molecular mechanisms involved, including modulation of bile salt synthesis, biotransformation/depuration of cholestatic compounds, changes in transporter expression/activity, and evocation of signaling pathways.

Keywords: Bile formation, cholestasis, silymarin, hepatoprotection, bile salt metabolism, hepatocellular transporters, lithocholate, estrogen.

1. INTRODUCTION

Derivatives of milk thistle (*Silybum marianum*) have been used as herbal remedies for almost 2000 years and, in particular for the treatment of hepatobiliary diseases, since the 16th century. From that time onwards, its fruits and seeds have been empirically used as a folk medicine for the treatment of acute hepatitis, chronic liver disease, jaundice and gallstone symptoms, among others. In an attempt to better understand its hepatoprotective properties, animals studies has been carried out since 1949, when Eichler and Hahn found that a milk thistle tincture had beneficial effects against trinitrotoluene- and CCl_4-induced hepatotoxicity [1]. These experimental studies, and further confirmatory investigations in patients with liver disease, prompted its commercialization in the pharmaceutical market as silymarin (SIL), an standardized extract from milk thistle seeds, in 1969 [2].

Despite SIL has been widely used in clinical practice, the mechanisms underlying its hepatoprotective properties remain largely unknown. This is in part because SIL has been successfully (and safely) used in an empiric manner in patients for centuries, which explains the priority given to clinical trials in patients with different hepatopathies before a clear picture of the manner the drug exerts beneficial effects has emerged. The recent comprehension of this conceptual deficit prompted a number of experimental studies either *in vivo*, using experimental animals, or *in vitro*, using isolated hepatocytes or hepatocellular lines in culture. This greatly helped not only to better understand how SIL actually attenuates hepatocellular injury in human hepatopathies but also to envisage new therapeutic applications in liver disease.

In this review, we addressed a number of recent experimental studies showing a novel feature of SIL, namely: its anticholestatic properties in experimental models of hepatocellular cholestasis with clinical correlate. These findings may contribute to explain the beneficial effects of SIL on liver diseases where a cholestatic component exists, e.g. in the cirrhotic liver. This may also help to extend its applications to other liver diseases where pure, hepatocellular cholestasis occurs, such as many forms of drug-induced cholestasis.

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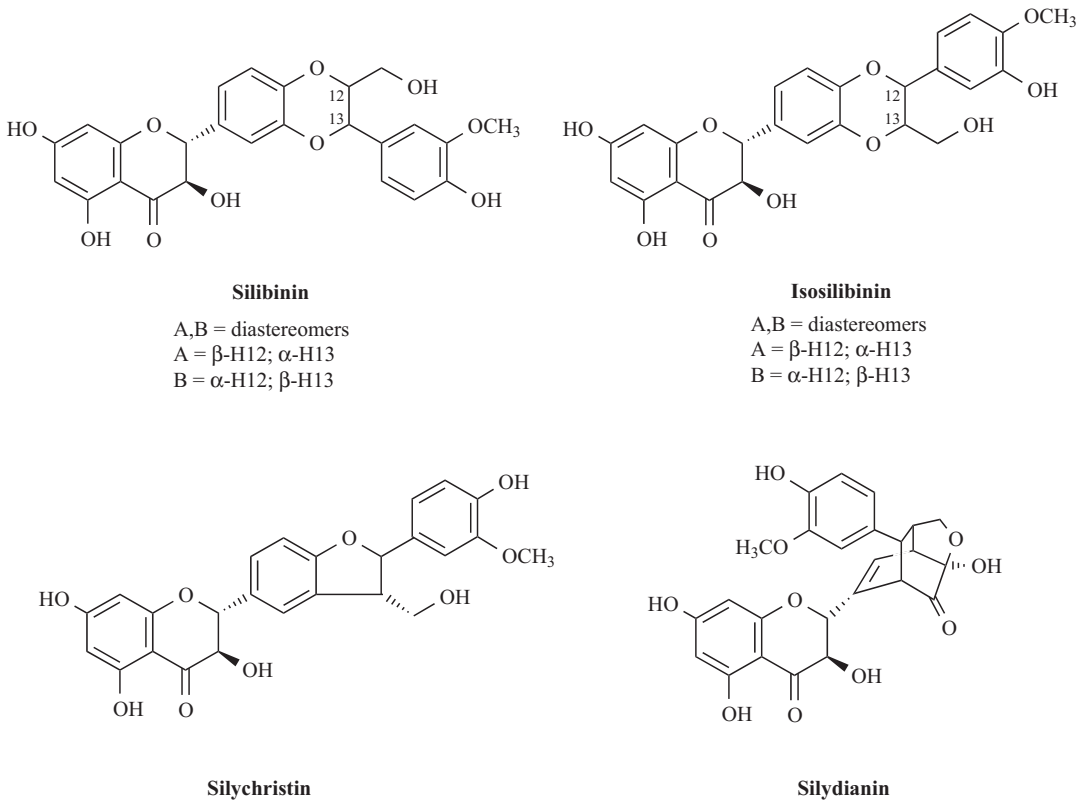

Fig. (1). Chemical structures of the main constituents of silymarin, namely: silibinin, isosilibinin, silydianin and silychristin. These compounds possess a phenylchromanone skeleton (flavonoid moiety), with different oxidative links to a molecule of coniferyl alcohol (lignan moiety), so that it can be classified as flavonolignans. Silibinin and isosilibinin are both present as a pair of diastereomers, epimeric at C12 and C13.

2. SIL AS A HEPATOPROTECTIVE AGENT

2.1. Chemistry of SIL

SIL is a standardized extract from fruits and seeds of milk thistle (*Silybum Marianum* (L.) Gaertn). It is mainly composed of silibinin (SB, also referred to as "silybin"), isosilibinin, silydianin and silychristin [3]; SB and isosilibinin are both present as a pair of diastereomers, epimeric at C12 and C13 (Fig. **1**). All these constituents possess a phenylchromanone skeleton (flavonoid moiety), with different oxidative links to a molecule of coniferyl alcohol (lignan moiety). Therefore, they can be classified as flavonolignans. The four flavonolignans are synthesized by the thistle from a common, unknown precursor during fruit maturation [4].

The standardized SIL extract contains approximately 70% SIL, the remaining being a chemically not well-defined fraction, composed mostly of polymeric and oxidized polyphenolic compounds [5]. The flavonolignan, SB, which represents 60-70% of SIL, has been identified as the major biologically active component [6,7]. SIL should therefore not be considered as an "alternative medicine" nowadays, as its composition is now chemically well defined.

Since SIL has a relatively poor intestinal absorption, efforts have been made to increase its oral bioavailability by combining its active component, SB, with phosphatidyl-choline (compound dB 1016) [8], with liposomes containing variables amount of cholesterol and phospholipids [9], or with lipid microspheres formed by an internal oily core, surfactants (e.g., soybean lecithin) and different cosurfactants (Span® 20, Tween® 20 or 80, and propylene glycol) [10]. All these formulations show higher oral bioavailability and facilitate passive targeting to the liver, thus conferring greater pharmacological activity compared with pure SB or SIL.

2.2. Therapeutic Applications of SIL

SIL became formally acknowledged as a hepatoprotective therapeutic agent in 1969, when it was lunched into the pharmaceutical market, supported by experimental findings both in experimental animals and humans. Since then, SIL became commercially available in more than 100 countries for the treatment of liver diseases, and 10-15% of patients attending liver disease clinics in the United States reported having taken milk-thistle derivatives, mostly without advise of a physician [11].

More than one-hundred clinical, cooperative studies in humans were conducted to evaluate SIL therapeutic efficiency in liver diseases of various etiologies. They included acute viral hepatitis, drug- and toxin-induced hepatitis, alcoholic liver disease, and chronic hepatitis or cirrhosis. Unfortunately, some of these studies are flawed by inadequate experimental designs, incorrect patient selection and monitoring criteria, or underpowered statistical analysis. Nevertheless, many of them allow valid conclusions to be drawn. In those patients where SIL showed a beneficial action, at least one of the following favorable effects were reported: *i*) improvement of biochemical markers of liver

function (transaminases, gamma-glutamyltransferase, bilirubin, alkaline phosphatase, albumin and prothrombin time, among others), *ii*) amelioration of hystological alterations, *iii*) acceleration of the recovery, or *iv*) improvement of survival. Effectiveness of SIL in clinical trials has been the subject of several recent reviews, and we refer the readers to them for a critical discussion of the results obtained [12-16]. Shortly, evidences for improvement were most compelling for alcoholic liver disease, as histological findings, liver transaminase levels and prothrombin time were consistently reported as improved in trials dealing with this disease. In alcoholic liver cirrhosis, SIL significantly improves biochemical markers and reduces liver-related mortality. On the contrary, and in spite of some positive results in patients, no valid conclusions can be drawn in acute or chronic viral hepatitis. The same holds true for nonalcoholic fatty liver disease, where some non-controlled data showed that SB reduces insulin resistance, liver steatosis and plasma markers of liver fibrosis. Finally, the available trials in patients with toxic liver diseases (e.g., those induced by exposure to halogenated hydrocarbons, toluene or xylene) or by iatrogenic liver diseases (e.g., those induced by antipsychotic or tacrine administration) have limited therapeutic value, since they are mostly outdated and underpowered. Therefore, final evidence of SIL efficacy in these hepatopathies awaits better-designed trials. Regarding this, the National Center for Complementary and Alterative Medicine, in collaboration with the National Institute of Diabetes and Digestive and Kidney Disease, is launching phase I/II trials aimed to define optimal dosing regimens, and to identify patient cohorts and surrogate markers for assessment of SIL efficacy in nonalcoholic steatohepatitis and chronic hepatitis C [11].

None of the numerous clinical studies published so far has reported any adverse effect of SIL, nor harmful interactions with other drugs. There is also no evidence to date of any danger for SIL intake during pregnancy or lactation. This is in line with animal studies showing that no embrionic or fetal lesions, nor teratogenic effects, is observed when SIL is administered during the critical phase of organogenesis, even at doses as high as 2,500 mg per Kg of body weight [17]. Similarly, neither perinatal nor postnatal toxicity was observed in either mothers or offspring when SIL was administered at this high dose during the last third of pregnancy, and up to the end of lactation [17].

2.3. Hepatoprotective Mechanisms of SIL

The molecular mechanisms explaining hepatoprotective properties of SIL have been the subject of considerable interest in recent years. However, many points remain to be explored. The advances in molecular and cellular biology add nowadays enormously to our understanding of the pathophysiology of many hepatopathies, and will greatly contribute to find new horizons for these investigations.

The main findings to date were obtained in both whole animals and *in vitro* models of hepatocellular function. In many of them, the active component of SIL, SB, rather than the whole purified extract, has been used. These studies have consolidated the concept that SIL hepatoprotective effect is multifactorial. The mechanisms of hepatoprotection confirmed so far are:

1. *Protein synthesis-inducing properties.* SIL induces activation of RNA polymerase I in the nuclei of hepatocytes, thus increasing the synthesis rate of rRNA [18]. This enhances the biosynthetic apparatus in the cytoplasm, thus leading to an increase in synthesis rate of both structural and functional proteins. At least conceptually, this stimulation may enable cells to counteract loss of transporters and enzymes occurring under many pathological conditions. Protein induction may also stimulate hepatic regeneration in hepatopathies leading to loss of normal parenchyma.

2. *Antioxidant properties.* SIL protects against oxidative stress-induced hepatocellular damage due to its own scavenging properties against certain reactive oxygen species. Its active component, SB (as dihemisuccinate), is a strong scavenger of hydroxyl anion (OH^-) and hydroxyl hypochlorous acid (HOCl), a powerful antibacterial but also a cytotoxic agent produced by neutrophils [19]. SB-phosphatidylcholine complex (IdB 1016) scavenges lipodienyl and other carbon-centered free radicals (e.g., methyl and tricloromethyl radicals) [20]. On the other hand, SB has a far lower scavenging effect on superoxide anion (O_2^-), and has no reaction with hydrogen peroxide (H_2O_2). In addition, SB enhances endogenous antioxidant defenses, like those mediated by superoxide dismutase [21] and the glutathione system [22]. Overall, these effects protect hepatocytes against free-radical-induced lipid peroxidation of membranes and the concomitant membrane breakdown induced by several toxic, pro-oxidant agents, such as ethanol [21,23], CCl_4 [24], acetaminophen [22,25], and iron [26], among others. The latter protective effect may be in part due to SIL ability to complex iron [26]. Similarly, the bile salts (BSs) retained in cholestatic disorders due to the secretory failure are though to produce in part damaging effects by inducing oxidative stress [27,28], and SIL may counteract these harmful effects. This has been recently corroborated in experimental bile-duct ligation, where SIL improves the antioxidant status of obstructed livers and prevents lipid peroxidation [29,30]. Oxidative stress is a major feature of alcoholic and non-alcoholic liver steatosis, and a key determinant of their progression to cirrhosis. The finding that SIL protects against these hepatopathies (see above, item 2.2) may be therefore causally associated with its free-radical-scavenging properties. Protective mechanisms at the level of free-radical production in these diseases are less likely. For example, SIL (and its active component, SB) failed to inhibit the alcohol-inducible, cytochrome P450 2E1 (Cyp2E1), which metabolizes alcohol and other molecules to generate radical oxygen species [31].

3. *Membrane-stabilizing properties.* SIL is a membranotropic agent, which stabilizes cellular membranes by either a direct effect on

physicochemical membrane properties or by indirect biochemical mechanisms, modulating membrane lipid metabolism and composition. SIL has a direct, stabilizing effect against osmotic-stress-induced hepatocellular damage [32], and against BS-induced cytolysis [33]. BSs accumulated in cholestasis are though to actively incorporate cholesterol and phospholipids into their hydrophobic core, thus inducing extensive membrane damage, although BS ability to induce oxidative stress has been shown to be another deleterious factor. We have shown that SIL counteracts membrane alterations induced by other micelle-forming detergents, like Triton® X-100 [34], ruling out the possibility that the protective effect in BS-induced cytolysis is only due to antioxidant properties of SIL against BS-induced oxidative stress. SIL may also influence membrane lipid composition by inhibiting synthesis of cholesterol [35] and of certain phospholipids, such as phosphatidylcholine and phosphatidylethanolamine [36]; this reduces membrane turnover and improves membrane integrity. Disorders in membrane stability and lipid metabolism are a common feature in liver disease, and the stabilizing properties of SIL may help to counteract the harmful effect of toxic agents that affect membrane fluidity, such as galactosamine [37] and CCl_4 [24]. In addition, both SIL and its active component, SB, fluidize microsomal membrane by incorporation into the hydrophobic-hydrophilic interface of the microsomal bilayer, which perturbs lipid structure by influencing packing of acyl chains [38]; rigidification of microsomal membrane is a common event in cholestasis, which can critically affect activity of microsomal enzymes [39].

4. *Antifibrogenic properties.* SIL inhibits fibrogenesis in experimental hepatopaties [40,41]. This effect is likely to be mediated by inhibition of stellate-cell proliferation, and its further transformation into myofibroblasts. This diminishes expression of the profibrogenic procollagen alpha1(I) and the tissue inhibitor of metalloproteinases-1, most likely by down-regulation of the pro-fibrogenic cytokine, TGF-β1 [42,43]. Kupffer cells promote stellate-cell proliferation and activation, and counteracting action of SIL at this level may play a key role. When administered at concentrations reached in plasma after clinical doses, SB inhibits production by Kupffer cell of mediators involved in stellate-cell activation, such as reactive oxygen species and leukotrienes [44]. Inhibition of leukotriene production is due to inhibition of 5-lipoxygenase, the enzyme that catalyzes leukotriene formation from arachidonic acid. Interestingly, 5-lipoxygenase inhibition was shown to lead to Kupffer cell growth arrest and apoptosis [45]. Production of leukotrienes by granulocytes was also inhibited by SB [46]. Antifibrinogenic properties of SIL may help to explain its antifibrotic properties in biliary cirrhosis secondary to biliary obstruction in rats [40] and, perhaps, the protective effect on parenchymal alterations and portal inflammation observed in patients with chronic hepatitis [47]. By doing so, SIL may aid to slow down progression of certain chronic hepatopathies into cirrhosis, which is the primary determinant of morbidity and mortality in patients with chronic liver disease.

5. *Signaling-evoking properties.* SIL is a potent, *in vitro* inhibitor of cyclic AMP (cAMP)-phosphodiesterase [48], which catalyzes degradation of intracellular cAMP; thus, an increase in the hepatic cAMP levels induced by SIL is likely, and may act as a second messenger of some beneficial effects of SIL (e.g., stabilization of cellular membranes).

6. *Antidoting effect against Amanita phalloides poisoning.* SIL inhibits uptake of α-amanitin, the amatoxin from the poisonous mushroom, *Amanita phalloides*, by competing with its basolateral transport system [49]; this blocks entry of the amatoxin into the hepatocyte, and prevents inhibition of RNA polymerase II, and the concomitant blockage of protein synthesis.

3. CHOLERETIC AND ANTICHOLESTATIC PROPERTIES OF SIL

A comprehensive understanding of the modulatory effects of SIL on bile secretion under normal conditions, and its beneficial effects in cholestasis, requires to address preliminarily both the physiological mechanisms of bile formation and the current advances in mechanisms of cholestasis. In this section, these issues will be covered at some level of detail before choleretic and anticholestatic properties of SIL are discussed.

3.1. Physiological Mechanisms of Bile Formation

Bile formation is an osmotic process driven by the vectorial transport of certain solutes into bile. For a solute to induce blood-to-bile water transport primarily, it needs to be secreted in a quantitatively significant amount, and to be actively concentrated and retained in a confined space (the bile canaliculus). Once secreted, these solutes induce passive water movement in response to osmotic gradients *via* both paracellular and transcellular routes. From the multiple solutes composing bile, only BSs, HCO_3^- and glutation, either reduced (GSH) or oxidized (GSSG), are thought to fulfill these requirements. Water movements driven by these solutes are facilitated by the water channels, *aquaporins* (AQP) type 9 and 8, localized at the basolateral and apical membranes of the hepatocyte, respectively [50].

As expected for any solute exerting osmotic forces at the canalicular level, a linear relationship between BS secretion and bile flow has been observed in all the vertebrate species examined, including man. This bile-flow fraction is conventionally referred to as "BS-dependent bile flow" (BSDF) [51]. Since a positive value is in most cases obtained when extrapolating BS output to zero, a BS-independent bile flow (BSIF) is also apparent, which has been attributed (so far) to both HCO_3^- and GSH output. This primary (canalicular) secretion is further modified by cholangiocytes during its transit along bile ducts, as a result of a balance between secretin-dependent fluid secretion, and the obligatory absorption of water, electrolytes and organic solutes [52,53]. Fig. **2** depicts a schematic representation of

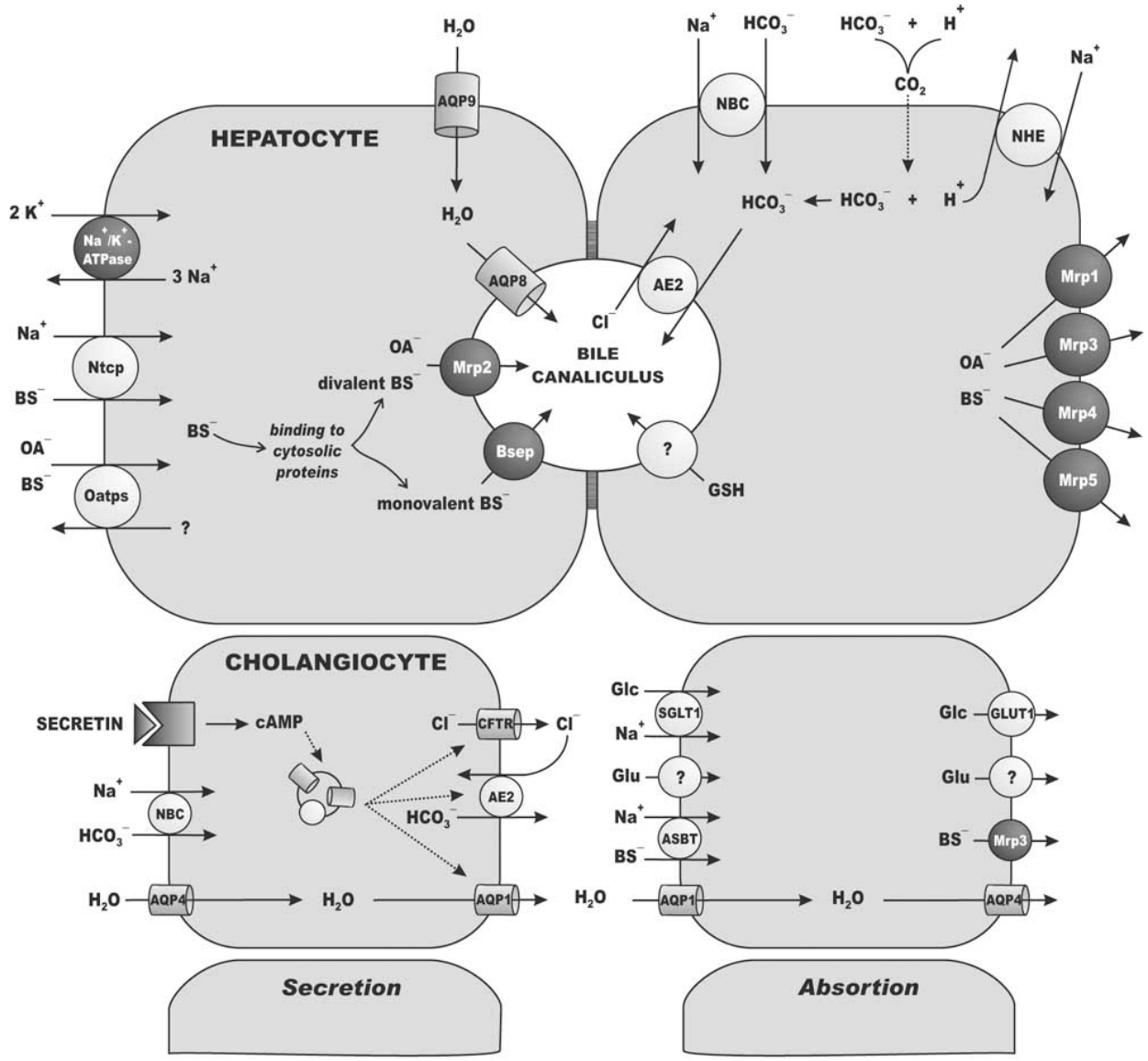

Fig. (2). Schematic representation of transport proteins directly involved in bile flow generation, both in hepatocytes and cholangiocytes. ATP-dependent transporters are depicted as black circles. Na^+-dependent bile salt (BS) uptake at the hepatocyte sinusoidal level is mediated by Ntcp, which is driven by the electrochemical Na^+ gradient generated and maintained by the pump, Na^+/K^+-ATPase. The Na^+-independent hepatic uptake of organic anions (OAs) and BSs is mediated by members of the Oatp family. After uptake, BSs bind to cytosolic proteins prior to canalicular excretion, which is primarily mediated by Bsep (for amidated, monoanionic BSs) or by Mrp2 (for divalent, bipolar, sulfated or glucuronidated BSs). Mrp2 also mediates transport of other non-BS OAs, like bilirubin. The basolateral membrane also possesses several transporters belonging to the Mrp family (Mrp1, Mrp3, Mrp4 and Mrp5) which, although normally expressed at low levels in normal liver, are up-regulated in cholestasis to compensate for reduced Mrp2/Bsep expression, thus maintaining ongoing OA and BS efflux from the hepatocyte. GSH is also excreted into the canaliculus through Mrp2 (with low affinity) and, predominantly, *via* an as yet unidentified, high-affinity canalicular GSH transporter. HCO_3^- is excreted into the bile canaliculus by an array of active and passive transport mechanisms. The Na^+ gradients generated by Na^+/K^+-ATPase allow for basolateral efflux of H^+ *via* NHE. Once extruded, H^+ neutralizes plasma HCO_3^-, enabling subsequent passive diffusion as CO_2. Plasma HCO_3^- can be also taken up by NBC. At the canalicular pole, AE2, is responsible for HCO_3^- excretion. Osmotic gradients created by the solutes actively secreted and retained in the bile canaliculus induce water transport across both the sinusoidal and the canalicular membranes *via* the water channels, AQP9 and AQP8, respectively. Canalicular bile is modified by cholangiocytes during its transit along bile ducts. This modification comprises secretin-dependent output of a HCO_3^--rich fluid (involving AE2 and driven by Cl^- gradients maintained by CFTR) and obligatory water absorption (driven by osmotic gradients created by absorption of electrolytes and organic solutes). The organic solutes reabsorbed are glucose (Glc, taken up by SGLT1, and exported by GLUT1), glutamate (Glu, *via* as yet unidentified transport systems), and BSs (taken up by ASBT, and exported by Mrp3). Blood-to-bile water movement at the ductular level is facilitated by the presence of constitutive AQP4 in the basolateral membrane, and secretin-stimulated AQP1 in the apical membrane. Secretin induces apical transport activity of AE2, CFTR and AQP1 by elevating cAMP, which stimulates exocytic insertion of transporter-containing periapical vesicles, available on demand.

the main solutes and transporters relevant to bile flow formation at both hepatocellular and cholangiocellular levels.

BSs are the predominant organic solutes in bile. Their concentration is about 1000-fold higher in bile than in portal blood [54]. The main sinusoidal transport system for BS uptake is the *Na^+-taurocholate cotransporting polypeptide*, which has been isolated and cloned from both rat liver (Ntcp, Slc10a1) [55] and human liver (NTCP, SLC10A1) [56]. Ntcp/NTCP are glycoproteins of 362 and 349 amino acids, respectively, which are driven by a transmembrane Na^+ gradient maintained by the Na^+/K^+-ATPase pump, which is also strategically localized in the sinusoidal membrane [57]. Ntcp accounts for the transport of more than 80% of amidated BSs (the major circulating BSs), and only 40% of their unconjugated, parent compounds [58]. The remaining fraction is taken up by a non-electrogenic, Na^+-independent transport system, mediated by the *organic anion-transporting polypeptides* (Oatp/OATP for rat and human, respectively) [54]. In addition to conjugated and unconjugated BSs, Oatp/OATP accepts a wide range of amphipathic, organic compounds including bilirubin, leukotrienes, estrogens and an elevated number of exogenous organic anions (e.g., bromosulfophthalein) [59]. The driving force for this carrier-mediated uptake has not been established as yet, but may involve exchange with intracellular HCO_3^- or GSH [60]. Four OATPs have been cloned and characterized in human liver (OATP-A, OATP-B, OATP-C and OATP-8), and three were identified in rats (Oatp1, Oatp2 and Oatp4) [61,62].

Under physiological conditions, BSs are excreted mainly as C24 amides, conjugated with either glycine or taurine. BS transport across the canalicular membrane is the rate-limiting step in its overall blood-to-bile transfer. BS canalicular transport is mainly mediated by the *bile salt export pump* (Bsep, Abcb11; also referred to as *sister of P-glycoprotein*), an adenosine triphosphate (ATP)-binding cassette (ABC) transporter [61,62]. In human liver, BSEP is a 160-kDa protein which exhibits high affinity for BSs [63]. In contrast to monoanionic BSs, canalicular efflux of divalent, bipolar sulfated or glucuronidated BSs is mediated by the *multidrug resistance-associated protein 2* (Mrp2) [54,64,65].

A candidate to partially account for BSIF is HCO_3^-. A role for HCO_3^- was formerly supported by the observation of Hardison and Wood [66] that bile flow is reduced in isolated rat livers perfused with a buffer solution where HCO_3^- had been replaced with tricine. However, it remains questionable whether a bile-to-perfusate gradient of HCO_3^- can be maintained, due to the high ion permeability *via* the paracellular pathway [67,68]. Nevertheless, situations have been reported in which an increase in BSIF was only associated with elevations in HCO_3^- output, e.g. following cGMP administration [69]. Although both passive and active mechanisms are involved in HCO_3^- hepatocellular blood-to-bile transport, overall this process is active in nature, since depends ultimately on Na^+/K^+-ATPase activity. This pump maintains low intracellular Na^+ activity, so that the plasma-to-cytosol Na^+ gradients allow for basolateral efflux of H^+ *via* the *Na^+/H^+ exchanger* (NHE, isoforms 1 and 4 in hepatocytes) [70]. Once extruded, H^+ neutralizes plasma HCO_3^-, enabling subsequent passive diffusion as CO_2 [66]. Plasma HCO_3^- can be also taken up by the Na^+/HCO_3^- cotransport system localized in the basolateral membrane [71]. At the canalicular pole, the Cl^-/HCO_3^- countertransporter, *anion exchanger 2* (AE2), is responsible for HCO_3^- excretion into the bile canaliculus [72].

BSIF is also determined by GSH/GSSG excretion. These peptides are secreted into bile at relatively high concentrations (5-10 mM), mainly in its reduced form (~80%). Changes in bile flow parallel GSH excretion, when modified by administration of different pharmacological agents [73] or, more directly, by administration of GSH itself, its monoethyl ester derivative or any of its three amino-acid components [74].

Hepatocellular GSH transport mechanisms are poorly understood. The liver is the main site of GSH synthesis, exporting this peptide into both blood and bile. Most, if not all, biliary GSH comes from this intracellular source, as no uptake of GSH has been detected at physiological plasma concentrations [75]. The transporters involved in GSH canalicular secretion have been only characterized on the basis of kinetics evidences, but no canalicular transporter has been cloned so far. However, a high affinity, electrogenic carrier has been functionally characterized [76-79]. This transport system exports actively reduced GSH into bile, and can transfer GSSG and GSH conjugates as well, although with lower affinity. Another transporter likely involved in GSH canalicular transport is Mrp2. However, this carrier bears low-affinity towards GSH, although it can transfers GSSG and GSH-conjugates with high affinity [77].

Canalicular bile flow is further modified by cholangiocytes lining the bile ducts by both secretory and absorptive processes [52,53]. Fluid secretion is driven by the secretin-regulated output of a HCO_3^--rich fluid, driven by the Cl^-/HCO_3^- exchange system, AE2 [72]. The driving force for AE2 activity is the out-to-in concentration gradient of Cl^-. This gradient is maintained by Cl^- efflux across the apical membrane. This efflux is mediated by the *cystic fibrosis transmembrane regulator* (CFTR), and driven by the intracellular, negative electrical potential [80]. Blood-to-bile water movement at the ductular level is facilitated by the presence of constitutive AQP4 in the basolateral membrane, and secretin-stimulated AQP1 in the apical membrane. Secretin stimulates apical transport activity of AE2, CFTR and AQP1 by elevating cAMP, which induces coordinated, exocytic insertion of pre-formed transporters, localized in a vesicular, periapical compartment available on demand [81]. On the other hand, obligatory water absorption occurs, which is driven by osmotic gradients created by the bile-to-plasma transport of electrolytes and organic solutes. This absorptive component would account for the net absorption of ductular water in interprandial periods, when plasma secretin levels are low. Absorbed organic solutes comprise glucose (*via* the apical, Na^+-dependent transporter, SGLT1, and the basolateral, facilitative transporter, GLUT1) [82], glutamate (*via* as yet unidentified Na^+-dependent and Na^+-independent uptake systems) [83], and BSs (taken up by the *apical Na^+-dependent BS transporter*, ASBT, and returned back into plasma by the basolateral export pump, Mrp3, and probably by a truncated form of ASBT, *t*-ASBT) [84-86]. The relative contribution of canalicular and ductular bile flow has major species-related differences. Bile flow is largely canalicular in

rodents [87], whereas it is evenly divided among BSDF, BSIF and ductular bile flow in humans [88].

3.2. Pathophysiological Mechanisms of Acquired Hepatocellular Cholestasis

Acquired hepatocellular cholestasis can be defined as a partial or total impairment in bile flow formation due to non-congenital causes. This alteration may be caused by dysfunction of one or more transport events, leading to impairment of the biliary output of solutes actively involved in bile flow generation. Alternatively, bile flow failure can be a consequence of alterations in tight-junctional permeability, which impairs canalicular retention of solutes *after* excretion, with subsequent dissipation of osmotic gradients.

3.2.1. Impairment of Hepatocellular Transport Activity

Conceptually, any impairment in one or more transport steps involved in the hepatobiliary transfer of an endogenous solute identified to be choleretic should lead to cholestasis. However, the impact that these alterations will have on bile formation depends on whether this transport event is rate-limiting in the overall hepatobiliary transfer. If not, residual transport capability of the altered transporter should be reduced to a sufficient extent to become the rate-limiting step itself.

The physiological rate-limiting step in the overall transfer of solutes from blood into bile is their transport across the canalicular membrane. Furthermore, kinetics values for the canalicular transfer of both BS- and non-BS organic anions suggest that they are saturated under basal conditions *in vivo* [89]. Therefore, any reduction in the number of transporters expressed in the canalicular membrane are expected to noticeably impair bile secretory function. It is therefore rational that most of the recent works assessing mechanisms of hepatocellular cholestasis focus on alterations at this level.

Changes of hepatobiliary transporter expression/activity in acquired cholestasis may occur at a transcriptional, post-transcriptional or post-translational level, or a combination of these. These alterations result in changes in transporter activity in terms of days, hours or minutes/seconds, respectively.

Transcriptional and post-transcriptional alterations of hepatobiliary transporters. Changes in expression of transport systems at a transcriptional level involves impairment in mRNA synthesis, whereas changes at a post-transcriptional level can be due to mRNA instability, augmented mRNA degradation, or defective translation of mRNAs into proteins. These long-term regulations can occur in cholestasis as a primary consequence of the cholestatic injury or, more commonly, as a consequence of secondary, adaptive changes aimed to minimize deleterious consequences of the initial insult. For example, in several animal models of experimental cholestasis, there are common patterns of responses at the level of transporter expression that helps to partially protect the hepatocyte from retention of toxic compounds, particularly BSs and bilirubin [90]. These changes comprise down-regulation of basolateral uptake systems, such as Ntcp [91,92], Oatp-1 [93,94] and Oatp-c [95], and up-regulation of basolateral export pumps, such as Mrp1 [96], Mrp3 [97-99], Mrp4 [100,101] and Mrp5 [97]. A somewhat similar pattern of expression of carrier proteins was shown to occur in patients with progressive familial intrahepatic cholestasis, with down-regulation of basolateral uptake systems (NTCP, OATP1B1 and OATP1B3), and up-regulation of basolateral export systems (MRP4) [101]. All these changes protects the liver from BS and bilirubin retention either by preventing uptake or by facilitating extrahepatic routes of excretion (e.g., urine) [62]. Contrarily, other changes can be regarded as detrimental rather than adaptive. For example, Mrp2 expression is consistently impaired in different experimental models of chronic cholestasis [102], whereas Bsep expression is by far less inhibited [103]. Studies made in patients with cholestasis failed to show a decrease in Mrp2 expression [104], suggesting that this transporter can be differentially regulated in humans.

Many of the changes in both basolateral and canalicular transporter expression are mediated by the coordinated activation of a number of nuclear hormone receptors, which act as transcription factors that translocate from the cytoplasm to the nucleus on binding a relevant ligand. It is therefore not surprising that activation of these transcription factors has beneficial effects in experimental models of cholestasis [95,105-107]. They comprise: *farnesoid X receptor* (FXR), the *pregnane X receptor* (PXR), the *constitutive androstane receptor* (CAR), the *vitamin D receptor* (VDR), the *retinoic acid receptor* α (RARα) and the *small heterodimer partner* (SHP), among others; the first four ones heterodimerize with the *retinoid X receptor* (RXR), enabling high-affinity DNA binding and further activation of gene transcription [62].

Activation of nuclear receptors in cholestasis can also induce adaptive changes in metabolizing enzymes involved in synthesis and depuration/inactivation of BSs. PXR and FXR activation refrains BS synthesis by repressing genes encoding for the rate-limiting, microsomal cytochrome P450 enzyme, cholesterol 7α-hydroxylase (Cyp7a1) [108]. Another gene relevant to downregulation of BS biosynthesis by BSs is sterol 12-α hydroxylase (Cyp8b1). This gene encodes for the cytochrome P450 enzyme catalyzing the hydroxylation of the 12α position of 7α-hydroxycholesterol, leading to cholate (C) formation; this increases the C-to-chenodeoxycholare (CDC) ratio, which affects the overall hydrophobicity of the BS pool. Similar to Cyp7a1, the Cyp8b1 promoter contains a negative BS response element, which can be targeted by FXR-induced SHP for negative interference of transcriptional activity [108]. In addition, PXR and FXR [109,110], together with CAR [111] and VDR [112], enhance expression of dehydroepiandrosterone sulfotransferase (Sult2a1), an enzyme involved in BS sulfation. These four nuclear receptors also enhance expression of cytochrome P450 3A (Cyp3a), a group of isoenzymes involved in BS detoxification *via* hydroxylation reactions [113-117]. Both sulfation and hydroxylation reduce BS hydrophobicity, and increase BS affinity for exporting transporters, thus decreasing cytotoxicity and favoring urinary excretion [118,119].

Post-translational alterations of hepatobiliary transporters. Intrinsic transport activity of hepatobiliary transporters can be impaired by direct inhibition by cholestatic drugs or, indirectly, by unbeneficial changes in

lipid microenvironment. Alternatively, alterations in dynamic localization of the transporters can occur, as a consequence of endocytic internalization.

A number of compounds, including cyclosporin A [120], glibenclamide [120], rifamycin [120], rifampicin [120], bosentan [121] and triglitzone [122], *cis*-inhibit ATP-dependent taurocholate transport in isolated rat liver canalicular membrane vesicles. This was confirmed for human BSEP for some of these compounds [123]. On the other hand, some cholestatic compounds *trans*-inhibit Bsep, as they exert inhibitory effects once transported into the canalicular lumen. For example, the Mrp2-substrate, cholestatic compound, estradiol 17β-D-glucuronide (E217G) has been suggested to *trans*-inhibit Bsep in rat liver, since it requires intact Mrp2-translocating activity to exert inhibitory effect [120]. However, Mrp2 inhibitory modulation of Bsep activity via, for example, protein-protein interactions cannot be excluded.

Another key determinant of carrier activity is the restraint exerted by the lipid microenvironment. Rigidification of plasma membrane, where transporters are embedded, may impair solute transport by constraining conformational changes required for the transporter translocating activity [124]. Changes in membrane fluidity and/or composition of the canalicular membrane domain occur under a number of cholestatic conditions, and were linked causally to biliary-secretory failure. They include bile-duct-ligation [125], or administration of cyclosporin A [126] and lithocholate (LC) [127], among others. Other cholestatic compounds, such as 17α-ethynylestradiol (EE), modify selectively fluidity and lipid composition of the sinusoidal membrane [128]. However, a role for plasma membrane rigidification in cholestasis is controversial, since some rigidifying agents (e.g., spironolactone) induce choleresis [129], and some fluidizing agents (e.g., organic solvents) induce cholestasis [130].

Impairment of transporter activity can also occur by carrier endocytic internalization, which induces relocalization of the transporters into intracellular vesicular structures, presumably the subapical, endosomal compartment. If maintained with time under chronic cholestatic conditions, sustained internalization may lead to delivery of the protein into the lysosomal compartment, followed by degradation [131]. Endocytic retrieval has been shown to occur in several models of experimental cholestasis for the canalicular, ABC transporters, Mrp2 and Bsep. They include cholestatic maneuvers like bile-duct ligation [131], hyperosmotic perfusion [132,133] and oxidative challenge [134,135]. This retrieval also occurs after administration of the cholestatic compounds, lipopolysaccharide [133], $E_2$17G [136,137], taurolithocholate (TLC) [138,139] and cyclosporin A [140]. This pathomechanism also was shown to occur for Mrp2 in acquired, cholestatic hepatopathies in humans, including primary biliary cirrhosis [104] and antidepressant-induced cholestasis [141]. In the latter case, redistribution towards the basolateral membrane rather than into intracellular structures occurs, which would help to export bilirubin and other potentially toxic organic anions back to plasma.

3.2.2. Impairment of Tight Junctional Permeability

Tight junctions (*zonulae occludens*) seal the canalicular lumen between adjacent hepatocytes, thus providing a barrier to diffusion of biliary solutes from bile into blood. Changes in these structures under physiological or pathological conditions can occur due to molecular alterations leading to reduction in the number, density and/or width of the strands forming the tight-junctional complex [142,143]. This is often accompanied by disarrangement of tight-junctional-associated proteins, like ZO-1 [144], and of other components contributing to barrier function, like occludin [145]. These structural changes parallel with functional alterations, characterized by regurgitation of biliary constituents into plasma [146]. These functional variations can participate in the regulation of the biliary secretory function (if they are reversible and facultatively regulated by hormonal control) [147,148], or can represent a pathophysiological mechanism of cholestasis (if they are persistent and severe enough to impair irreversibly tight-junctional function) [149-151].

Impairment in tight-junctional permeability is a common feature in cholestasis. It occurs in bile-duct ligation [142,152] and under conditions of oxidative stress induced by both menadione [148,153] and *tert*-butylhydroperoxide [154,155]. It is also impaired in several models of drug-induced cholestasis, such as those induced by administration of $E_2$17G [148,156], cyclosporin A [148,157], acetaminophen [158], cholephilic dyes [159], carmustine [160], alpha-naphthylisothiocyanate [161] and LC [127], or its cholestatic conjugate, TLC [148,162]. In human hepatopathies, substantial alterations of tight-junctional proteins occur in primary biliary cirrhosis (predominantly in bile ducts) and in primary sclerosing cholangitis (predominantly in hepatocytes) [163]. This may explain the occurrence in these hepatopathies of high plasma levels of solutes confined otherwise to the biliary space, like hepatic enzymes (e.g., alkaline phosphatase) [164] or biliary lipoproteins (forming lipoprotein-X in plasma) [165].

3.3. Experimental Models of Cholestasis

Since the scope of this review focuses on the anticholestatic properties of SIL, and cholestasis induced by estrogens and monohydroxylated BSs have been studied so far to reveal SIL anticholestatic properties in experimental animals, we will describe in detail only the features of these experimental models.

3.3.1. Estrogen-Induced Cholestasis

Estrogen elevations occurring during the third trimester of pregnancy are thought to be a crucial contributor in the intrahepatic cholestasis occurring in pregnant, susceptible women [166]. Administration of estrogens as oral contraceptives or for postmenopausal replacement therapy were cholestatic for them as well [166]. Given these clinical implications, experimental cholestasis induced by estrogen administration in rodents has been widely used as an experimental model to assess the mechanisms involved in these cholestatic hepatopathies [166].

Long-term administration to rats of the synthetic estrogen, EE, reduces both BSDF and BSIF [167]. The

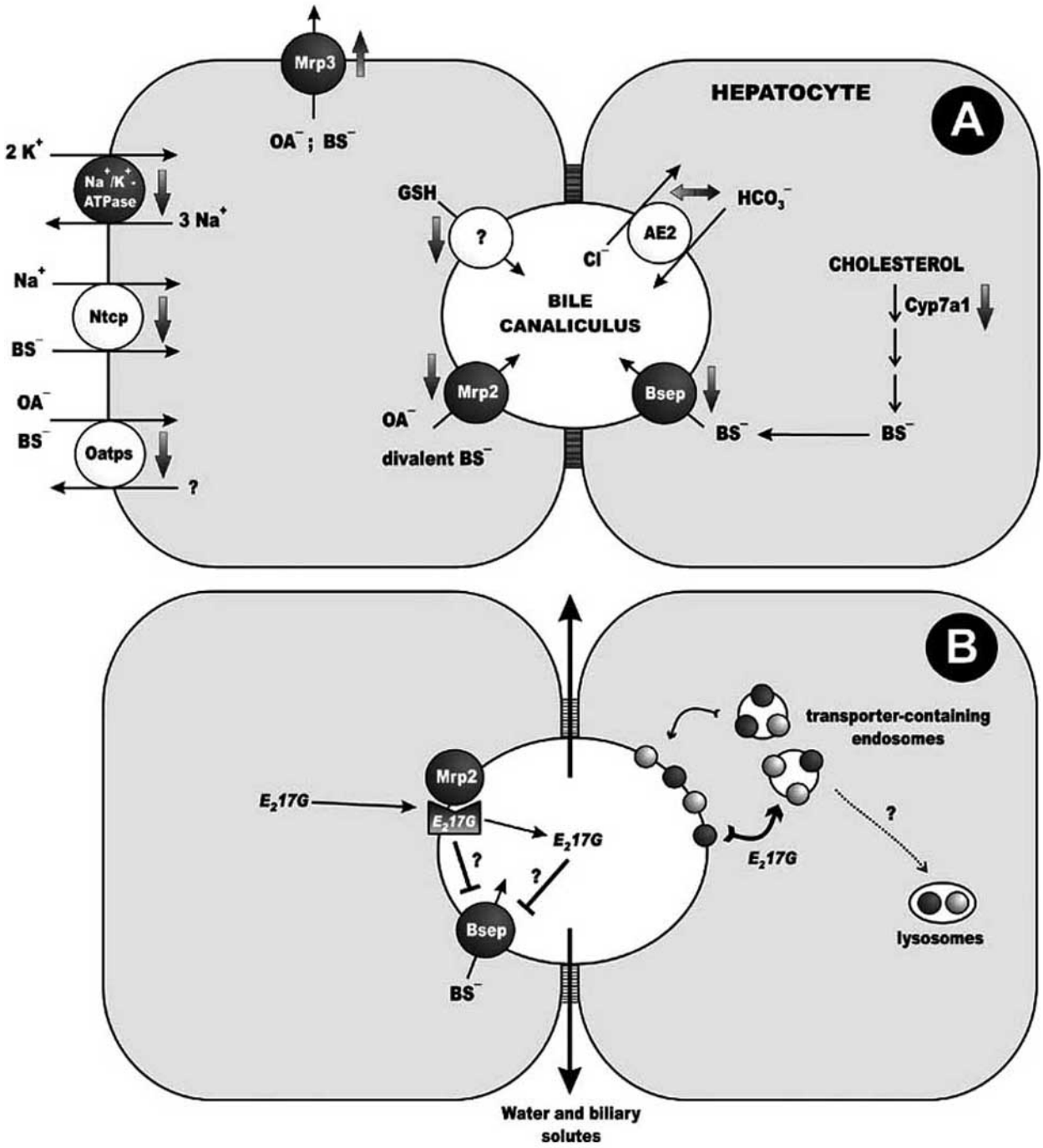

Fig. (3). Mechanisms of estrogen-induced alterations in bile formation. (*A*) Long-term administration of estrogens impairs expression and/or function of transport proteins. At the sinusoidal level, uptake of bile salts (BSs) and non-bile-salt organic anions (OAs) is impaired by down-regulation of Ntcp and Oatps, respectively. Na^+/K^+-ATPase activity, which is necessary for generation and maintenance of the Na^+ gradients required for Ntcp transport activity, is also impaired. At the canalicular level, transport of monoanionic BSs and OAs is affected as well, due to down-regulation of Bsep and Mrp2 expression, respectively. Increased Mrp3 exporting activity may contribute to reduce both BS and OA cytosolic levels, thus reducing availability for canalicular excretion. Biliary HCO_3^- secretion is also reduced, although AE2 expression is not affected by estrogen treatment. Impaired activity of Cyp7a1 inhibits BS synthesis, which plays a role in BS-output reduction. (*B*) Possible mechanisms responsible for the acute cholestatic effects of glucuronidated estrogen metabolites, such as estradiol 17β-D-glucuronide ($E_2$17G). Soon after administration, $E_2$17G induces reversible, endocytic internalization of Mrp2 and Bsep, which is associated with a diminished biliary output of OAs and BSs, respectively. Sustained transporter internalization may lead to lysosomal degradation of the endocytosed carriers. $E_2$17G requires Mrp2 transport activity to be cholestatic. It has been suggested that interaction between $E_2$17G and Mrp2, while the transporter is translocating the cholestatic agent, may affect somewhat Bsep transport function and localization. *Trans*-inhibition of Bsep by Mrp2-secreted $E_2$17G has been proposed as an alternative mechanism explaining Mrp2-dependency of $E_2$17G-induced cholestasis. Increased leakage of biliary components due to augmented paracellular permeability may also contribute to $E_2$17G-induced cholestasis.

mechanism involved is multifactorial, as shown in Fig. **3**, *A*. Alterations of the expression/activity of hepatocellular transporters are though to play a crucial role. EE decreases sinusoidal uptake of BSs [168] by inducing down-regulation of Ntcp [93,169,170] and Oatps (1, 2 and 4) [169] at a transcriptional level. Impaired expression of these transporters seems to be mediated by a diminution of the nuclear binding activity of trans-activators such as hepatocyte nuclear factor-1 (HNF-1), CAAT/enhancer binding protein (C/EBP) and PXR [169]. Down-regulation of BS uptake transport systems, together with augmented expression of basolateral export pumps like Mrp3 [106] are protective mechanisms which minimize hepatocellular accumulation of potentially toxic compounds, like BSs and bilirubin. However, augmented basolateral exportation of BSs may represent a causal factor accounting for BS secretory failure and cholestasis in this cholestatic model.

At the canalicular level, EE treatment decreases ATP-dependent, BS transport in canalicular membrane preparations [168]. This was associated with alterations in Bsep expression at a post-transcriptional level [170], a finding that has not been confirmed by others [106]. EE also impairs Mrp2 expression [102]; this decrease is not accompanied by a concomitant reduction in mRNA, indicating post-transcriptional regulation [102]. Because of the putative role of Mrp2 in BSIF formation by contributing to GSH excretion, this down-regulation may play a role in EE-induced cholestasis. However, this contention was challenged by studies showing that a similar absolute reduction in BSIF was obtained in normal and in Mrp2-deficient, TR^- rats [171]. It should be borne in mind that, unlike GSSG, GSH is a poor substrate for Mrp2, and more relevant transporters for GSH biliary excretion exist [77], which may be affected by EE as well. Another candidate to account for BSIF impairment is canalicular AE2, since HCO_3^- excretion is impaired in EE-treated rats [172,173]. However, neither AE2 expression nor activity is affected by EE [172]. It has been speculated that the impairment in HCO_3^- output might be caused by reflux of biliary HCO_3^- *via* leaky tight junctions. However, impairment of tight junctional permeability is not a confirmed event in EE-induced cholestasis, as no change [174], or slight changes [146] in this parameter were reported. Nevertheless, HCO_3^- is an easily permeant anion, and small changes in paracellular permeability may critically affect its bile-to-plasma gradient [175].

In addition to impairing hepatocellular transport systems, EE inhibits BS synthesis; this reduces endogenous BS pool and, consequently, BS output [176,177]. The overall change in BS content is accompanied by a relative enrichment of the BS pool in both β-muricholate (β-MC) and the secondary BS derived from its intestinal conversion, hyodeoxycholate (HDC). On the other hand, a decrease in the relative contribution of C and its secondary BS, deoxycholate (DC), is apparent [173,178]. These quantitative and qualitative alterations are caused by the coordinated action of post-transcriptional down-regulation of the microsomal enzyme, Cyp7a1 [176,179,180], and the simultaneous lack of inhibition of the mitochondrial enzyme, sterol 27-hydroxylase (Cyp27a1); Cyp7a1 catalyses the key, rate-limiting step of the *classical (neutral)*, major pathway of the overall BS synthesis, whereas Cyp27a1 catalyses the first step in the *alternative (acidic)* pathway of BS synthesis. The latter pathway produces selectively CDC and those BSs derived from its further hepatic and/or intestinal conversion, i.e., MC and HDC [176]. Preferential formation of BSs belonging to the CDC group is reinforced by the simultaneous inhibition of sterol 12α-hydroxylase (Cyp8b1), the key enzyme regulating biosynthesis of the BSs belonging to the C group [176].

Part of the alterations induced by EE on Cyp7a1 activity were attributed to the rigidifying effect of the cholestatic on the microsomal membrane, as Cyp7a1 is critically affected by changes in its lipid environment [179]. Compounds known to reverse the rigidifying effect of EE on microsomal membranes, such as Triton WR-1339 [179] and *S*-adenosyl-L-methionine [181], counteract both the interaction of EE with microsomal membranes and the impairment in Cyp7a1 activity. The effects of EE on lipid membranes are not limited to microsomes. EE reduces plasma membrane fluidity as well, by affecting selectively the basolateral domain [128]. This factor was implied in the impairment induced by EE in Ntcp activity and in Na^+-K^+-ATPase function, a key motor for the Na^+-dependent, Ntcp-facilitated BS uptake [182]. However, spironolactone, which induces similar plasma membrane rigidification to EE, induces choleresis rather than cholestasis [129].

Early events in estrogen-induced cholestasis were studied by administration to rats of the 17β-glucuronidated, endogenous estradiol metabolite, $E_2$17G. Potential hepatocellular targets for $E_2$17G cholestatic actions are depicted in Fig. **3**, *B*. Unlike its non-conjugated, parent compound, $E_2$17G administration to female rats produces rapid, dose-dependent, reversible cholestasis [183]. The bile flow decrease is due to an impairment in both BSDF [184] and BSIF [183]. The mechanisms by which these alterations occur are poorly known. $E_2$17G increases tight-junctional permeability [148,156], a factor that seems to be involved in the impairment in GSH excretion induced by the cholestatic, associated with *cis*-inhibition of its canalicular transporter [185]. In addition, evidences for $E_2$17G-induced, short-term changes in ABC transporter localization were obtained by our group [136]. Western blot and confocal analysis of Mrp2 content in intracellular membranes revealed partial internalization of Mrp2 into pericanalicular and intracellular vesicular structures during the acute phase of cholestasis, which is reversed by exocytic re-insertion during the recovery period. These structural changes parallel alterations in bile flow and in the biliary secretion of the model substrate, dinitrophenol-*S*-glutathione, suggesting a causal link between internalization and cholestasis. The recovery, but not the acute alteration induced by $E_2$17G in Mrp2 localization and function, is critically dependent on microtubule integrity [186]. A similar correlation between localization and transport activity was reported by us for Bsep, as studied both *in vivo* and in isolated rat hepatocyte couplets (IRHCs) [137]. Interestingly, administration of $E_2$17G to Mrp2-deficient, TR^- rats induces neither intrahepatic cholestasis [187] nor Bsep endocytic internalization [137]. $E_2$17G also fails to inhibit Bsep when the protein is expressed in a Sf9 insect cell line, unless Mrp2 is coexpressed [187]. To explain these unexpected results, it was suggested that interaction between the cholestatic agent and Mrp2 while the cholestatic is being translocated by this

transporter may be a key event in $E_2$17G-induced cholestasis, by altering somewhat Bsep transport function and localization [187]. *Trans*-inhibition of Bsep by Mrp2-secreted $E_2$17G has been proposed as an alternative mechanism to explain dependency on Mrp2 of $E_2$17G-induced cholestasis [120]. However, this mechanism seems not to be crucial, as no decrease in bile flow occurrs in isolated, perfused livers of TR^- rats, even when the $E_2$17G concentrations achieved in bile were equivalent to those reached in normal, Wistar rats [187]. Whatever the mechanism involved, these studies point endocytic internalization of relevant canalicular transporters as a key pathomechanism explaining bile flow impairment in $E_2$17G-induced cholestasis. These findings may also help to explain the loss of canalicular transporters in chronic estrogen-induced cholestasis, since sustained internalization stimulus may lead to increased transporter lysosomal degradation.

3.3.2. Monohydroxylated BS-Induced Cholestasis

The monohydroxylated BS, LC, and its taurine- and glycine-conjugated derivatives, TLC and glycolithocholate, represent a small fraction of the total BS pool both in humans [188] and rats [189]. However, their levels increase in several human hepatopathies, where they may either initiate or perpetuate the hepatic failure. Indeed, monohydroxylated BSs have been suggested to play a role in the liver dysfunction occurring in primary biliary cirrhosis [190], familial intrahepatic cholestasis type 1 (Byler disease) [191], total parenteral nutrition-induced cholestasis [192] and neonatal cholestasis [193].

Administration of either LC or TLC to laboratory animals is a useful model of monohydroxylated BS-induced cholestasis. TLC induces acute and reversible cholestasis in rodents, with a bile flow nadir at 15-20 min after administration [194]. The recovery from LC-induced cholestasis is highly dependent on biliary disposal of the cholestatic itself, and on its conversion into less toxic metabolites, *via* Cyp3a-mediated hydroxylation [195]. This phase I metabolism involves preferentially 6β-hydroxylation, with formation of the non-cholestatic BSs, murideoxycholate (MDC) and β-MC [196,197].

When administered acutely, TLC impairs both BSDF and BSIF [198]. The mechanisms underlying these alterations are still poorly understood. Fig. **4A** schematizes some possible molecular targets involved in TLC acute cholestatic effect.

TLC induces selective damage of canalicular membranes, leading to loss of microvilli, increase in cholesterol content and further membrane rigidification [199-201]. These alterations may affect water permeability of the canalicular membrane, contributing to the bile flow impairment [199]. TLC also induces multiple transport alterations at the canalicular level. It impairs canalicular transport of BSs [139,202], and that of the Mrp2 substrates, bromosulfophthalein [203] and dinitrophenol-*S*-glutathione [138]. Biliary excretion of GSH is also decreased [138]. Like $E_2$17G, TLC decreases the density of Mrp2 [138] and Bsep [139] in the canalicular membrane, and increases the occurrence of intracellular vesicles containing these transporters. The exact mechanism by which TLC induces this retrieval has not been ascertained as yet. Recent lines of evidence suggest that modulation of protein kinase C (PKC) isoforms by TLC could be involved. TLC activates the novel PKC isoform, PKCε, in isolated hepatocytes [204]. In line with this, dependency on PKC activity of the protective effect of TUDC and MC, another anticholestatic BS, was observed by our group for Bsep transport activity [205]. Wortmannin, a pan-specific phosphoinositide 3-kinase (PI3K) inhibitor, prevents the arrest of the transcytotic vesicular pathway, the impairment of the biliary output of the Mrp2 substrate, dinitrophenol-S-glutathione, and the decrease in bile flow induced by TLC [206]. This suggests a role for PI3K in TLC-induced cholestasis. Furthermore, the effect of TLC on PKCε would be mediated by this signaling pathway [206].

In addition to impairing hepatocellular transport activity, TLC also alters tight-junctional permeability [148]. Although a direct effect of TLC itself cannot be ruled out, TLC-induced PKC activation is likely to be involved, since PKC activation induces cholestasis by increasing tight-junctional permeability [147].

Monohydroxylated BSs fail to induce cholestasis and liver damage when administered chronically at repeated doses having cholestatic effects when administered alone [207]; higher doses may induce hepatotoxicity rather than cholestasis [113,116]. Accelerated metabolic conversion to non-toxic BSs, and adaptive changes in hepatobiliary transport play a crucial role in this refractoriness (see Fig. **4B**). Transcription factors like PXR [113], CAR [116] and FXR [208,209] are critically involved in this phenomenon. LC-induced activation of PXR results in repression of Cyp7a1 (which blocks BS biosynthesis), up-regulation of Cyp3a (which favors LC metabolization), and over-expression of Oatp2 (which can operate reversibly by exporting BSs at the sinusiodal level) [113]. Activation of CAR reinforces Cyp3a and Oatp2 induction, and up-regulates the basolateral export pump, Mrp3 [116]. Finally, LC-induced activation of FXR reinforces Cyp7a1 repression and mediates inhibition of the expression of transporters involved in BS uptake, such as Ntcp and Oatp1 [210].

3.4. Effect of SIL on Normal Biliary Secretory Function

SIL is a cholephilic compound. After being taken up by hepatocytes, the flavonolignan is partially glucuronidated, and further excreted into bile [211]. Due to its multiple modulatory effects on hepatic cells (see above, item 2.3), it is not surprising that SIL influences bile secretory function as well.

When administered intraperitoneally to male Wistar rats for 5 days, SIL induces a dose-dependent, stimulatory effect on bile flow and BS output, with a maximum stimulatory effect at a dose of 100 mg/kg body weight [212]. The choleretic effect of SIL is accounted for by a selective stimulation of BSDF, without any measurable effect on BSIF. BSDF elevation is mainly due to stimulation of the BS synthesis rate, as an increase in the size of the endogenous BS pool occurs, without any change in the expression of the ileal BS transporter, ASBT [173], another key determinant of the BS pool size. Similarly, no change in the transport capability of the canalicular BS transport system, Bsep, is recorded *in vivo*, as the maximal biliary secretion (*T*m) of the model, non-toxic BS, TUDC, is not influenced by SIL administration; since Bsep is the rate-limiting step in the

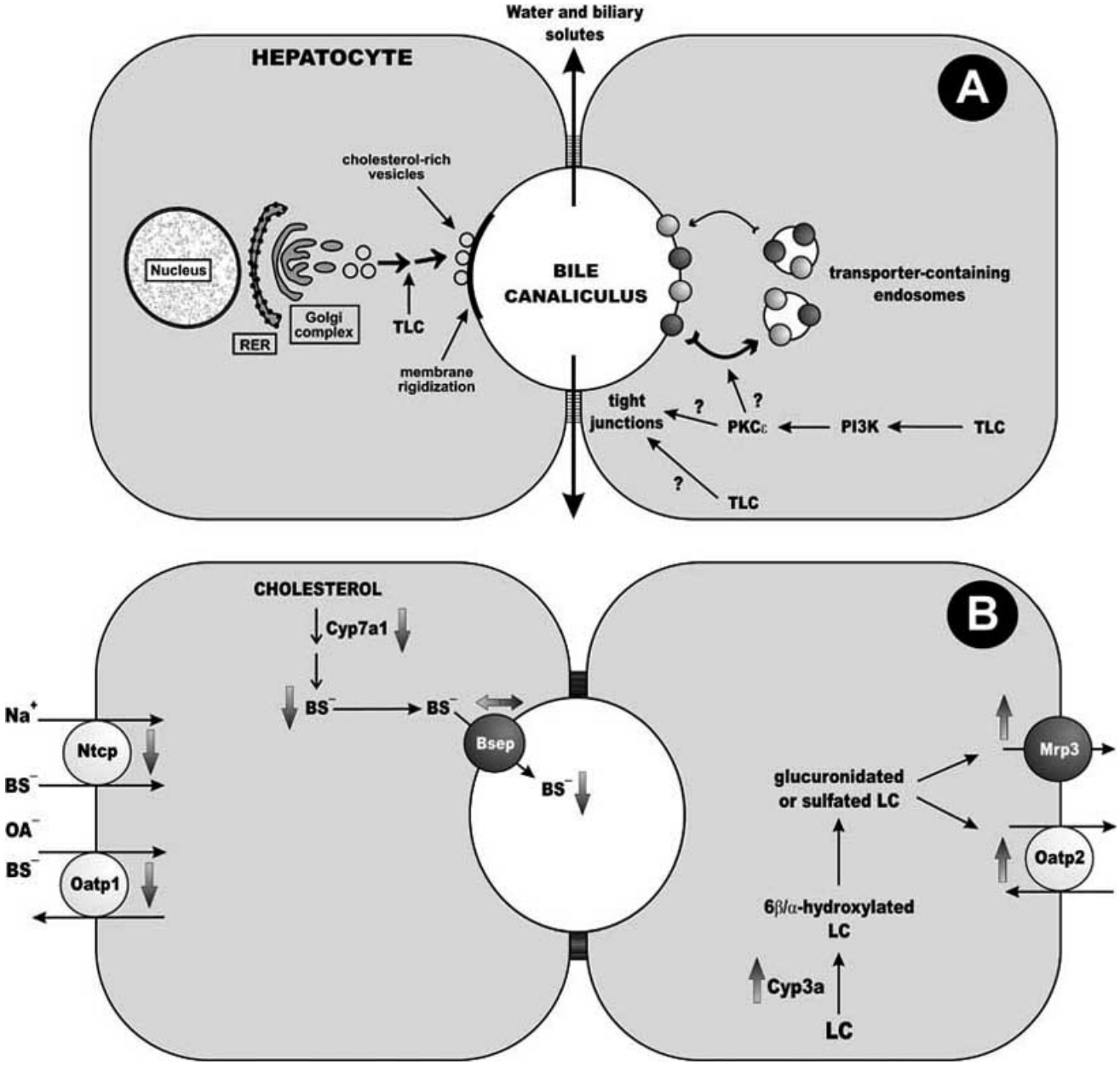

Fig. (4). Possible mechanisms responsible for the cholestatic effects of monohydroxylated bile salts. (*A*) Mechanism involved in short-term effects of taurolithocholate (TLC) after acute administration. Rapid delivery of cholesterol-rich vesicles to the canalicular membrane induced by TLC leads to rigidification of this membrane domain, which may cause a decrease in water membrane permeability, and the consequent impairment in bile flow. Decreased canalicular transporter activity due to membrane rigidification is also likely. Retrieval of canalicular transporters like Mrp2 and Bsep also plays a crucial role in the acute cholestatic effect induced by TLC. The activation of PKCε, *via* phosphoinositide 3-kinase (PI3K) signaling, could shift the targeting/retrieval balance of transporter-containing vesicles towards retrieval, leading to internalization of canalicular transporters. Increment of tight-junctional permeability may be mediated by both a direct or a PKC-dependent effect of TLC. (*B*) Adaptive changes justifying absence of cholestasis during chronic lithocholate (LC) administration. Intracellular accumulation of potentially toxic bile salts (BSs) is prevented by down-regulation of sinusoidal transporters involved in BS uptake, such as Ntcp and Oatp1. Cyp7a1 is also down-regulated, resulting in diminished BS synthesis. Both events contribute to the decreased biliary BS output. Induction of Cyp3a contributes to LC detoxification by forming more hydrophilic, non-cholestatic meabolites. Up-regulation of the sinusoidal transporters, Mrp3 and Oatp2, helps to export conjugated cholestatic meabolites back to plasma.

handling of BSs by the liver, *T*m of TUDC reflects maximal Bsep transport activity [212].

The mechanisms by which SIL increases the amount of circulating BSs remain speculative, but the analysis of the individual BSs that are stimulated by SIL provides some clues. Fig. **5** depicts the biosynthetic pathways that could be stimulated by SIL. Possible enzymatic control points, based upon the changes recorded in BS composition, are also depicted. A key control point in BS synthesis is Cyp7a1, the microsomal enzyme responsible for cholesterol 7α-hydroxylase activity. This enzyme catalyses the key, rate-limiting step of the *classical*, major pathway of the overall BS synthesis, i.e. the formation of BSs belonging to both C and CDC groups [213,214]. However, these BSs are not

elevated to the same extent by SIL. Approximately 90% of the increment is accounted for by CDC and other metabolically-related compounds, such as α-MC, β-MC, ursodeoxycholate (UDC) and HDC. This suggests preferential stimulation of the biosynthetic branch leading to CDC and/or inhibition of 12α-hydroxylase activity (Cyp8b1), the enzyme that catalyzes the conversion of the common C and CDC precursor, 7α-hydroxy-4-cholesten-3-one, leading to irreversible C formation [214]. Whether inhibition of 12α-hydroxylase activity, if present, occurs *via* FXR/SHP, a well-established suppressor of Cyp8b1 transcriptional activity [108], remains to be ascertained. Additional stimulation of the alternative (acidic) pathway of BS synthesis, which is regulated by mitochondrial sterol 27-hydroxylase (Cyp27a1) and that leads selectively to formation of BSs belonging to the CDC group, should also be considered.

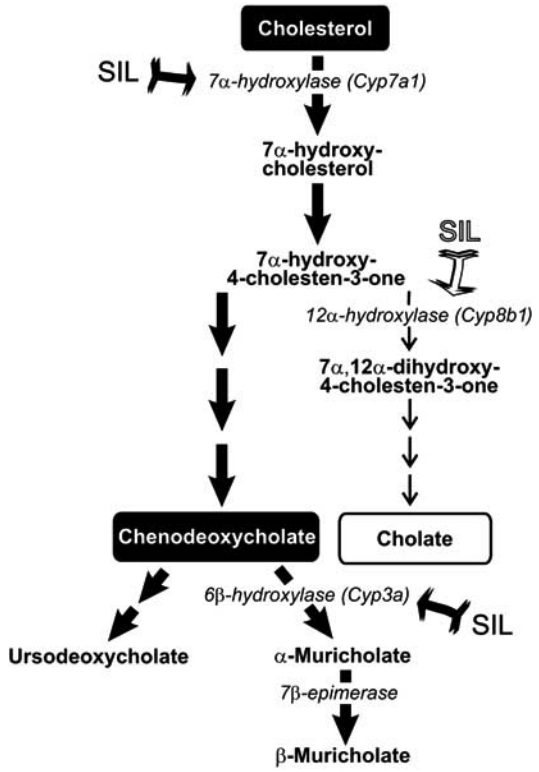

Fig. (5). Proposed effects of silymarin (SIL) on the *classical* biosynthetic pathway of bile salt synthesis. The biosynthetic branch preferentially stimulated by SIL has been highlighted with thick arrows. Putative enzymatic targets stimulated by this flavonolignan are indicated with filled arrows, whereas possible inhibitory effects are shown with empty arrows. Since SIL increases the total amount of bile salts in the endogenous bile salt pool, stimulation of Cyp7a1, which represents the rate-limiting step in the overall synthesis of bile salts from cholesterol, is apparent. Preferential enrichment of the bile salt pool in chenodeoxycholate and other bile salts derived from its further metabolic conversion suggests a selective stimulation of this biosynthetic branch. Inhibition of Cyp8b1, which catalyzes conversion of the common cholate and chenodeoxycholate precursor, 7α-hydroxy-4-cholesten-3-one, leading to irreversible C formation, could also be involved. Since SIL increases the biliary amount of muricholate (formed by 6β hydroxylation of chenodeoxycholate) to a greater extent than that of chenodeoxycholate itself, it is proposed that 6β hydroxylase activity (due to Cyp3a) is stimulated by SIL as well.

A finding particularly relevant in terms of hepatoprotection is that administration of SIL increases the biliary content of BSs derived from the further conversion of CDC to more hydrophilic compounds, such as α- and β-MC, *via* Cyp3a-catalysed 6β-hydroxylation. This suggests that 6β-hydroxylating activity is stimulated by the flavonolignan. Similarly, the BS pool is enriched in UDC and HDC, two BSs situated downstream of CDC in the BS biosynthetic pathway. Interestingly, α-MC, β-MC and UDC [215], together with HDC [216], share hepatoprotective properties against the hepatocellular damage induced by more hydrophobic BSs, and against a variety of drug-induced, toxicological or cholestatic insults [217]. Whether enhancement of Cyp3a activity by SIL involves activation of at least one of the nuclear receptors that modulate positively the expression of this enzyme (i.e., FXR, PXR, CAR and VDR) is unknown at present.

3.5. Beneficial Effects of SIL in Animal Models of Cholestasis

The findings that, under normal bile secretory conditions, SIL induces choleresis and beneficial effects on biliary BS pattern by forming more hydrophilic, or even hepatoprotective BSs provided the rationale to assess the efficacy of SIL as an anticholestatic agent. Indeed, these changes resemble the adaptive modifications suffered in the hepatocelular BS biosynthetic machinery under cholestatic conditions, and therapeutic agents that favor this adaptivity bear anticholestatic properties as well (see above, item 3.2.1).

Although SIL effects on bile secretion may be beneficial in most cases of cholestasis primarily involving dysfunction of biliary secretion, the choleretic effect of SIL may be detrimental in obstructive cholestasis, where choleresis may exacerbate damage of biliary structures by increasing intrabiliary pressure. This is why SIL anticholestatic properties were tested in two models of experimental cholestasis affecting primarily bile secretory function. They comprise cholestasis induced by estrogens and cholestasis induced by the monohydroxylated BS, TLC, two cholestatic models with clinical correlate. A summary of the beneficial effects of SIL in these models of cholestasis, both at the level of BS metabolism and on function of transporters relevant to bile formation, is depicted in Fig. **6**.

3.5.1. Prevention of Estrogen-Induced Cholestasis by SIL

Because of its low toxicity, if any, and the absence of reports on teratogenicity, SIL would be a potential therapeutic candidate for the prevention/cure of pregnancy-induced cholestasis. The causal relationship of this hepatopathy with estrogens prompted us to assay the beneficial effect of SIL in an *in vivo* model of estrogen-induced cholestasis [173].

When coadministered for 5 days with EE, SIL prevented, in a dose-dependent manner, the reduction of both bile flow and BS output induced by the estrogen [173]. At a dose of 100 mg/kg body weight, which exerts maximal beneficial effects, SIL fully counteracted the decrease in BS output, whereas the impairment in the overall bile flow was only partially prevented, due to an only partial ameliorating effect on BSIF.

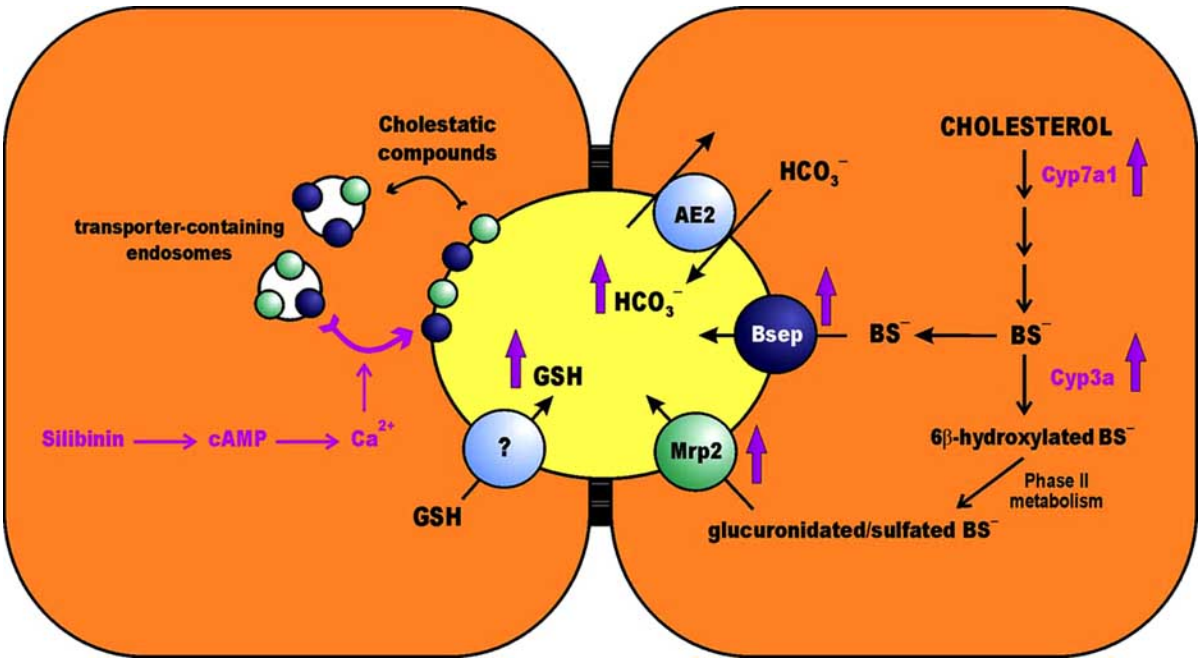

Fig. (6). Beneficial effects of silymarin, or that of its active component, silibinin, on both the hepatocellular transporter alterations and the bile salt (BS) biosynthetic defects occurring in cholestasis induced by estrogens and monohydroxylated BSs. Potential targets for silymarin action are indicated with thick arrows. Silymarin prevents the decrease in Bsep and Mrp2 transport function occurring in cholestasis, by mechanisms not involving changes in carrier expression. Silymarin increases the size of the endogenous BS pool by enhancing Cyp7a1 activity, and counteracts the decrease in overall BS synthesis induced in rats by a subacute (5-day) exposure to 17α-ethynylestradiol. Silymarin also accelerate the conversion of taurolithocholate into muricholate and murideoxycholate, by enhancing 6β-hydroxylation (Cyp3a activity); these 6β-hydroxylated, non-cholestatic metabolites can be more easily glucuronidated and sulphated, and excreted *via* Mrp2. When pre-administered in isolated rat hepatocyte couplets before acute exposure to estradiol 17β-D-glucuronide or taurolithocholate, silibinin significantly counteracts retrieval and consequent BS-secretory failure induced by the cholestatic compounds, *via* a cAMP-mediated, Ca^{2+}-dependent mechanism. Silymarin shows also protective effects against the decrease in the biliary output of glutathione (GSH) and HCO_3^-, two solutes involved in the generation of the BS-independent bile flow. Prevention of the decrease in GSH output was however far more effective in acute taurolithocholate-induced cholestasis; whether this was due to improvement in the function of GSH canalicular transport systems remains unknown. On the other hand, HCO_3^- output was significantly improved by silymarin in both models of cholestasis, although the mechanism/s involved remain to be ascertained (see text for details).

A key mechanism by which EE impairs BS output is inhibition of the overall BS synthetic pathway [176,177], a finding mainly attributed to the inhibitory effect of EE on Cyp7a1. SIL completely prevented the decrease induced by EE in the size of the endogenous BS pool produced by the inhibition of BS synthesis; this explains the improvement in BS output induced by SIL. This finding is in line with our above-mentioned results that SIL *per se* expands the BS pool size and stimulates the *de novo* BS synthesis rate [212]. Although the mechanism(s) by which SIL counteracts the effect of EE on BS synthesis cannot be anticipated with certainty, some possibilities can be hypothesized: *i*) increment of Cyp7a1 expression, due to SIL ability to enhance protein synthesis [18]; *ii*) increment of the microsomal membrane fluidity by incorporation of the flavonolignan to the hydrophobic-hydrophilic interface [218], which may prevent the unfavorable effects of EE on Cyp7a1 lipid microenvironment [179]. In line with the latter possibility, other compounds known to reverse the rigidifying effect of EE on hepatocyte lipid membranes, including *S*-adenosyl-L-methionine and the nonionic detergent, Triton® WR-1339, counteract the interaction of EE with microsomal membranes and/or the inhibition of Cyp7a1 activity [179,181]. Finally, our studies also revealed that Bsep transport activity, as assessed by the *T*m of the model BS, TUDC, is improved by SIL, thus contributing to the prevention of EE-induced impairment of BS output.

SIL also conteracts, although partially, the effect of EE on BSIF [173]. This is due, at least in part, to a partial restoration of HCO_3^- output and, to a far lesser extent, GSH output. The mechanism involved in the beneficial effect of SIL on HCO_3^- excretion is unknown, but does not involve AE2, as neither activity nor expression of the transporter is impaired by EE [172]. A protective effect at the level of tight-junctional permeability, thus preventing HCO_3^- paracellular reflux, is likely, and awaits experimental confirmation. Output of GSH is also impaired by EE. Since GSH output is thought to depend in part on Mrp2 transport activity, we assessed the influence of SIL pre-treatment on expression and function of this carrier. Mrp2 transport activity, as assessed by pharmacokinetics studies using the model substrate, bromosulfophthalein, is decreased by EE, and this alteration is extensively prevented by SIL [173]. Surprisingly, the marked down-regulation of Mrp2 expression induced by EE is not counteracted by SIL. These results emphasize a crucial role of alterations in the *functional status* rather than in the number of canalicular transporters in the cholestatic effect induced by EE, the former being efficiently counteracted by SIL. Whether these beneficial effects reflect modulatory changes in the

transporters themselves (e.g. phosphorylation status, binding to modulatory sites, etc.), or they occur at the level of the lipid microenvironment where these transporters are embedded, remains to be ascertained. Membranotropic properties of SIL (see above, item 2.3) suggest that the last possibility is likely. Finally, the fact that GSH output remains extensively impaired in rats coadministered with EE and SIL despite Mrp2 transport activity is extensively restored suggests that transporters other than Mrp2 are involved in EE-induced GSH secretory impairment, as suggested also by others [171], whose activity is not normalized by SIL.

SIL does not inhibit UGT2B1, the UDP-glucuronosyltransferase isoenzyme involved in formation of 17β-glucuronoconjugated EE [173]. This metabolite is thought to be a key mediator of the cholestatic manifestations induced by EE [219]. Thus, it is conceivable that SIL exerts its anticholestatic effect at a post-metabolic level, by counteracting the harmful effects of this metabolite. To test this hypothesis, we assessed the capability of SB, the major active component of SIL, to prevent the impairment induced by the model glucuronidated estrogen, $E_2$17G, in IRHCs by evaluating their ability to apically secrete the fluorescent BS analogue, cholyl-lysylfluorescein (CLF). Our results showed that SB counteracts the decrease in the percentage of couplets accumulating CLF in their canalicular vacuoles, in all the range of doses of $E_2$17G tested [173].

In further studies, we analyzed in IRHCs the ability of SB to counteract a major mechanism involved in the impairment of the BS secretion induced by $E_2$17G, namely: the endocytic retrieval of Bsep. SB almost completely prevented this alteration [220]. Therefore, this property could account, at least in part, for the ability of SIL to counteract estrogen-induced impairment of BS secretion.

A possible mechanism by which SB protects against estrogen-induced endocytic retrieval of Bsep is elevation of the second messenger, cAMP. This is supported by several lines of evidence: *i*) SB induces elevation of intracellular cAMP levels in isolated hepatocytes [220], an effect most likely due to its ability to inhibit cAMP- phosphodiesterase [48]; *ii*) like SB, cAMP prevents the endocytic retrieval of Bsep induced by $E_2$17G in IRHCs [137,220]; *iii*) SB shares with cAMP common downstream signaling events when preventing the impairment in BS canalicular transport; the protective effects of both SB and cAMP depend on Ca^{2+} elevations but not on protein kinase A (PKA) activation, as they are abolished by intracellular Ca^{2+} chelation but not by specific PKA inhibitors [220].

In summary, our results show that SIL is instrumental in preventing a wide range of cholestatic manifestations induced by estrogens both *in vivo* and in *in vitro* models. Its beneficial effects include total restoration of impaired BS output, which is mainly a result of SIL ability to counteract estrogen deleterious effects at the level of both BS synthesis and canalicular transport systems. In addition, SIL partially prevents the diminution of BSIF induced by estrogens, mainly by improving HCO_3^- output.

3.5.2. Prevention of TLC-Induced Cholestasis by SIL

Our finding that SIL facilitates metabolism of hydrophobic BSs to more hydrophilic, harmless BSs, prompted us to address the capability of the flavonolignan to counteract the cholestatic effect of the hydrophobic BS, TLC.

When administered for 5 days at a dose of 100 mg per kg body weight, SIL significantly prevented the development of cholestasis induced by a single, i.v. dose of TLC [221]. SIL pre-treatment partially prevented the decrease induced by TLC in both BSDF and BSIF. The latter effect is accounted for by an improvement in the biliary excretion of HCO_3^- and GSH, the two main determinants of this bile flow fraction.

Two mechanisms were found to be involved in the beneficial effect of SIL on TLC-induced cholestasis, namely: *i*) prevention of the transport alterations induced by TLC at the canalicular membrane level, and *ii*) bioactivation of metabolic pathways involved in TLC detoxification.

Endocytic internalization of canalicular transporters involved in bile flow generation, such as Mrp2 [138,222] and Bsep [139], occurs in TLC-induced cholestasis. We have showed that the SIL active component, SB, when co-administered acutely with TLC to IRHCs, prevents the rapid endocytic retrieval of Bsep, and the parallel impairment in the ability of IRHCs to accumulate apically the fluorescent BS analogue, CLF. Furthermore, we demonstrated that, such as occurs in $E_2$17G-induced cholestasis, SB anticholestatic effect involves, at least in part, cAMP as a second messenger, with Ca^{2+} elevation as the relevant downstream signaling event involved [220]. These experiments demonstrate that, apart from the beneficial effects of SIL involving changes in protein and lipid metabolism, which require long-lasting exposure to the hepatoprotector to occur, the flavonolignan exerts rapid, modulatory effects that counteract the cholestatic manifestations induced by TLC. Long-term and short-term effects of SIL may both act in concert. For example, subacute (5-day) administration of SIL *in vivo* increases the content of MCs and UDC in the endogenous BS pool [173], and these BSs have beneficial effects on TLC-induced cholestasis [205,223], including amelioration of Bsep function [205] and localization [205,222]. Other possible modulatory effects of SIL involve changes in composition/fluidity of the membrane domain where the transporters are inserted, as TLC increases the cholesterol content of the canalicular membrane, thus inducing membrane rigidification [199].

TLC detoxification after an *in bolus,* i.v. administration of the cholestatic agent involves rapid, metabolic conversion and further biliary excretion of their metabolites into bile, together with excretion of a small fraction of non-metabolized TLC. *In vivo* and *in vitro* studies show that 6β-hydroxylation, mediated by Cyp3a, is the predominant hepatic metabolic event involved, both in rats and mice. This leads to formation of non-cholestatic metabolites, like tauroconjugates of β-MC and MDC [196,197]. Furthermore, 6β-hydroxylation of LC favors LC 6-O-glucuronide formation, which avoids production of LC 3-O-glucuronide, an even more cholestatic compound than LC [224]. When administered subacutely for 5 days, SIL accelerated extensively the total amount of MDC excreted into bile immediately after TLC administration [221]. There was also a clear tendency towards increased biliary excretion of CDC, β-MC and HDC, three non-cholestatic BSs resulting from phase I metabolism of TLC. These results are in line with

our finding that SIL enhances 6β-hydroxylation of endogenous BSs (see above, item 3.4). These beneficial changes in BS metabolism and disposal resemble those induced by a number of anticholestatic, therapeutic agents with agonistic activity towards PXR, like rifampicin [225]. They are also similar to the adaptive changes induced by endogenous, hydrophobic BSs, when accumulated in cholestatic diseases [226]. Our finding that SIL shares with rifampicin this beneficial mechanism, if confirmed in humans, reinforces expectations on the use of SIL as an alternative anticholestatic agent to rifampicin, which, unlike SIL, is hepatotoxic to some extent [227].

4. CONCLUDING REMARKS

Despite extensive use of SIL as a hepatoprotective agent, its effects on normal bile secretion and its putative beneficial effects on cholestatic liver diseases affecting primarily bile secretory function (the so called "pure" cholestasis) had not been analyzed in detail until recently. Our laboratory has been actively engaged in covering these aspects. As a first step, we assessed the ability of SIL to modulate bile flow and BS secretion, using rats as an animal model [212]. The results obtained show that SIL exerts beneficial changes in BS metabolism. Indeed, SIL protective effects largely resembles that induced by anticholestatic agents like rifampicin and other ligands of transcription factors belonging to the orphan nuclear receptor family. They have in common their potential to activate metabolizing systems that favor conversion of hydrophobic BSs, accumulated by the secretory failure, into more hydrophilic ones. These BSs are less toxic, and more easily disposable *via* urine [228]. Whether SIL effect is mediated by activation of these transcription factors or by its fluidizing effect on microsomal membrane where these metabolizing systems are embedded, as suggested by *in vitro* experiments [38], remains to be ascertained. Lack of SIL effect on expression of carrier proteins when administered either alone or during cholestatic treatments suggests that the latter possibility is most likely. If so, SIL can represent an ideal complement to transcription-factor ligands used nowadays as therapeutic agents. It can even represent an alternative to non- and sub-responding patients, which may have congenital defective expression of these transcription factors. It should be borne in mind that counteracting the diminished protein levels of membrane-bound enzymes or transporters may not be sufficient to fully normalize catalytic/transport function when alterations in membrane lipid environment exist, such as occurs commonly in cholestasis [39]. Our finding that the impairment in Mrp2 transport function in EE-induced cholestasis is almost fully counteracted by SIL without preventing the decrease in Mrp2 membrane content [173] clearly illustrates the functional importance of this protective mechanism. Furthermore, SIL bears additional beneficial mechanisms that may be relevant to minimize cholestatic manifestations. One of them is its ability to prevent endocytic internalization of transporters in cholestasis, by modulating signaling events occurring downstream of cAMP [220]. Again, this property may complement the effect of inducers of carrier expression, by cooperatively assuring both proper localization and constitutive expression. Whether such a consorted, beneficial action of SIL will actually occur in patients with cholestatic hepatopathies remains to be ascertained, and this will represent a challenge for clinical researchers in their effort to develop better therapeutic strategies in liver disease. The exponential advances in cell and molecular biology applied to the understanding of the mechanisms of cholestasis and hepatoprotection will catalyze a constant feedback between basic research and applied therapeutics, aimed to achieve this ultimate goal.

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ABBREVIATIONS

SIL	=	Silymarin
SB	=	Silibinin
BS	=	Bile salt
cAMP	=	Cyclic AMP
Cyp	=	Cytochrome P-450 isoenzyme
GSH	=	Reduced glutathione
GSSG	=	Oxidized glutathione
AQP	=	Aquaporin
BSDF	=	Bile salt-dependent bile flow
BSIF	=	Bile salt-independent bile flow
AE2	=	Anion exchanger 2
CFTR	=	Cystic fibrosis transmembrane regulator
Ntcp/NTCP	=	Na^+-taurocholate cotransporting polypeptide
Oatp/OATP	=	Organic anion transporting polypeptide
Bsep/BSEP	=	Bile salt export pump
ABC	=	Adenosine triphosphate binding cassette
Mrp/MRP	=	Multidrug resistance-associated protein
IRHCs	=	Isolated rat hepatocytes couplets
NHE	=	Na^+/H^+ exchanger
AE2	=	Anion exchanger 2
CFTR	=	Cystic fibrosis transmembrane regulator
ASBT	=	Apical Na^+-dependent bile salt transporter
FXR	=	Farsenoid X receptor
PXR	=	Pregnane X receptor
CAR	=	Constitutive androstane receptor
VDR	=	Vitamin D receptor
RARα	=	Retinoic acid receptor α
SHP	=	Small heterodimer partner
RXR	=	Retinoid X receptor
C	=	Cholate
CDC	=	Chenodeoxycholate
$E_2$17G	=	Estradiol 17β-D-glucuronide
LC	=	Lithocholate

EE = 17α-ethynylestradiol

TLC = Taurolithocholate

MC = Muricholate

HDC = Hyodeoxycholate

DC = Deoxycholate

MDC = Murideoxycholate

PKC = Protein kinase C

TUDC = Tauroursodeoxycholate

PI3K = Phosphoinositide 3-kinase

UDC = Ursodeoxycholate

UGT = UDP-glucuronosyltransferase

CLF = Cholyl-lysylfluorescein

PKA = Protein kinase A

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