REVIEW

Ursodeoxycholic acid in cholestasis: linking action mechanisms to therapeutic applications

Marcelo G. ROMA, Flavia D. TOLEDO, Andrea C. BOAGLIO, Cecilia L. BASIGLIO, Fernando A. CROCENZI and Enrique J. SÁNCHEZ POZZI

Institute of Experimental Physiology (IFISE-CONICET), Faculty of Biochemical and Pharmacautical Sciences, National University of Rosario, Suipacha 570, S2002LRL Rosario, Argentina

ABSTRACT

UDCA (ursodeoxycholic acid) is the therapeutic agent most widely used for the treatment of cholestatic hepatopathies. Its use has expanded to other kinds of hepatic diseases, and even to extrahepatic ones. Such versatility is the result of its multiple mechanisms of action. UDCA stabilizes plasma membranes against cytolysis by tensioactive bile acids accumulated in cholestasis. UDCA also halts apoptosis by preventing the formation of mitochondrial pores, membrane recruitment of death receptors and endoplasmic-reticulum stress. In addition, UDCA induces changes in the expression of metabolizing enzymes and transporters that reduce bile acid cytotoxicity and improve renal excretion. Its capability to positively modulate ductular bile flow helps to preserve the integrity of bile ducts. UDCA also prevents the endocytic internalization of canalicular transporters, a common feature in cholestasis. Finally, UDCA has immunomodulatory properties that limit the exacerbated immunological response occurring in autoimmune cholestatic diseases by counteracting the overexpression of MHC antigens and perhaps by limiting the production of cytokines by immunocompetent cells. Owing to this multi-functionality, it is difficult to envisage a substitute for UDCA that combines as many hepatoprotective effects with such efficacy. We predict a long-lasting use of UDCA as the therapeutic agent of choice in cholestasis.

INTRODUCTION

UDCA (ursodeoxycholic acid) is currently the most widely used therapeutic agent for the treatment of hepatopathies of a cholestatic nature, and the only one approved by U.S. FDA (Food and Drug Administration) to treat PBC (primary biliary cirrhosis). Apart from cholestatic hepatopathies, its use is spreading to non-cholestatic liver

Key words: bile acid, cell death, cholestasis, hepatocellular transporter, immunomodulation, ursodeoxycholic acid (UCDA).

Abbreviations: ABC, ATP-binding cassette; AE2/Ae2, anion exchanger 2; ASBT/Asbt, apical sodium-dependent bile salt transporter; BSEP/Bsep, bile salt export pump; CDCA, chenodeoxycholic acid; CLT, cytotoxic T-lymphocyte; cPKC, Ca^{2+}-dependent protein kinase C; CYP3A4, cytochrome P450 family 3 subfamily A polypeptide 4; DISC, death-inducing signalling complex; $E_2$17G, oestradiol 17β-glucuronide; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ERK, extracellular-signal regulated-kinase; FADD, Fas-associated death domain; FAK, focal adhesion kinase; FasL, Fas ligand; FXR, farnesoid X receptor; HTL, helper T-lymphocyte; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-γ; IL, interleukin; LDH, lactate dehydrogenase; LFA-1, lymphocyte function-associated antigen-1; MAPK, mitogen-activated protein kinase; MDR/Mdr, multidrug-resistance protein; MPT, mitochondria permeability transition; MPTP, MPT pore; MRP/Mrp, multidrug resistance-associated protein; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor κB; NTCP/Ntcp, Na^+/taurocholic acid co-transporting polypeptide; OATP1/Oatp1, organic anion-transporting polypeptide 1; PBC, primary biliary cirrhosis; PFIC, progressive familial intrahepatic cholestasis; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PSC, primary sclerosing cholangitis; PXR, pregnane X receptor; ROS, reactive oxygen species; SRF, serum-response factor; tBid, truncated Bid; TCF, T-cell factor; TGF-β1, transforming growth factor-β1; TLCA, taurolithocholic acid; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; TRAILR-2, TRAIL receptor-2; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid; UGT1A1, UDP glucuronosyltransferase 1 family polypeptide A1.

Correspondence: Dr Marcelo G. Roma (email mroma@fbioyf.unr.edu.ar).

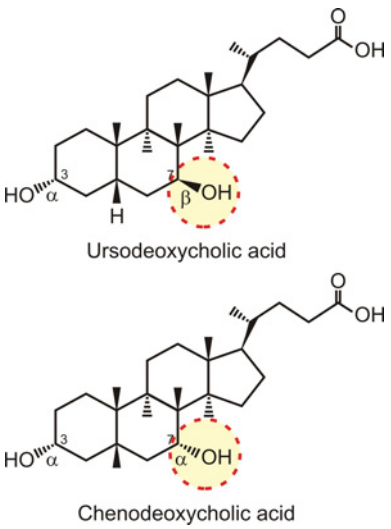

Figure 1 Chemical structures of UDCA (3α,7β-dihydroxy-5β-cholanoic acid) and its structural isomer CDCA (3α,7α-dihydroxy-5β-cholanoic acid)

The dotted circles denote the different orientation of the hydroxy group (-OH) in position 7 (β for UDCA and α for CDCA). This difference accounts for the marked dissimilarity in the hydrophobicity and membrane-damaging properties of both bile acids.

diseases, and even to non-hepatic ones. This exceptional versatility can only be understood on the basis of its multiple mechanisms of action. The sustained advance in the understanding of its several hepatoprotective properties gives us a unique opportunity to understand more deeply the relationship between the mechanisms of action of UDCA and its therapeutic applications.

UDCA has been used as part of a traditional Chinese medicine from the time of the Tang Dynasty (618–907 AD) for the treatment of jaundice, which employed the bile of black bears to cure several liver diseases. UDCA is the predominant bile acid in the bear and this is why it was named after 'urso' (bear in Latin). Its therapeutic use was rediscovered many years later by modern medicine, with the first reports on its use in Japan in 1961, followed by the publication of the first controlled trial in patients with PBC in 1989 [1].

From the chemical structural point of view, UDCA is 3α,7β-dihydroxy-5β-cholanoic acid, a bile acid with two hydroxy groups (-OH) at positions 3 and 7 in the cholane ring structure, with an α- and β-orientation respectively (Figure 1). The C-7 β-orientation confers the molecule a far higher hydrophilicity than that of its structural analogue with an α-orientation CDCA (chenodeoxycholic acid). This is why UDCA has much lower ability than CDCA to interact with, and disturb, lipid membranes. At the same time, it retains most of the regulatory beneficial properties of endogenous bile acids, for example their ability to activate regulatory signalling pathways or to trigger adaptive responses to bile acid overload [2,3].

MECHANISMS OF ACTION OF UDCA

Changes in the hydrophobicity index of the bile acid pool by UDCA treatment

In normal individuals, UDCA comprises no more than 4 % of the total endogenous bile acid pool. This value is increased to 40–60 % under a conventional dosage of 13–15 mg · kg^{-1} of body weight · day^{-1} [4], with virtually no change in total serum bile acids [5]. Replacement of potentially toxic hydrophobic endogenous bile acids in the total bile acid pool by an innocuous hydrophilic one would theoretically reduce bile-acid-mediated hepatic injury. However, this well-documented effect in normal individuals does not necessarily apply to every cholestatic condition. In PBC and PSC (primary sclerosing cholangitis), the pool size of primary bile acids (for example, cholic acid and CDCA) and secondary bile acids (for example, deoxycholic acid) do not change under UDCA treatment [6–8]. Only one study in early-stage PBC, with a normal deoxycholic acid pool size prior UDCA treatment, showed a reduction in the deoxycholic acid pool size [9]. Overall, these findings add little support for a significant role for the displacement of hydrophobic endogenous bile acids from the whole endogenous bile acid pool as a UDCA-protective mechanism in PBC and PSC. In contrast, in pregnancy-induced cholestasis, UDCA treatment displaces selectively free and taurine-conjugated cholic acid [10,11], a bile acid that is thought to play a role in intra-uterine fetal loss by acute anoxia [10]. However, other changes in bile acid composition induced by UDCA may be regarded as deleterious. For example, lithocholic acid levels increased in PSC patients whose progression was accelerated by high UDCA doses [8]; since lithocholic acid feeding promotes destructive cholangitis in mice [12], this might be a factor in this unfavourable outcome [13].

Apart from in serum, UDCA and its conjugates are enriched in bile, accounting for 19–64 % of total biliary bile acids, depending on the dosage [14]. This may be regarded as significantly protective for the biliary tree in cholangiopathies. Liver tissue is also enriched in UDCA at the expense of a reduction in more hydrophobic bile acids [15]. This may be a crucial factor in the therapeutic effects of UDCA in hepatocellular cholestasis involving selective bile acid transport at the canalicular level, as for example in responsive patients with early-stage PFIC (progressive familial intrahepatic cholestasis) type 1 (PFIC-1) and 2 (PFIC-2) [16].

Protection by UCDA against cell death induced by cytotoxic bile acids

Under normal conditions, cytosolic bile acids in hepatocytes and cholangiocytes are kept at levels below the critical micellar concentration, i.e. the minimal concentration from which bile acids display tensioactive

properties. In cholestatic hepatopathies, bile acids build up inside hepatocytes and, in the case of obstructive cholestasis, in cholangiocytes. CDCA and its amide conjugates are the endogenous bile acids that increase the most in chronic cholestatic hepatopathies [17], and thus are the main bile acids involved in the cholestatic damage [2].

It has been proposed that cytotoxic bile acids can differentially induce either necrosis or apoptosis depending on the severity of the cholestasis [18]; necrosis would be the major mechanism of cell death in severe cholestasis, whereas apoptosis would be the predominant one under milder cholestatic conditions [19].

Specific mechanisms of cellular protection against both apoptosis and necrosis have been described for UDCA, as summarized below.

Effect of UDCA on cytotoxic bile-acid-induced hepatocellular apoptosis

A strong association exists between hepatocellular apoptosis and cholestasis [20], and accumulated bile acids appear to play a key role [3,21]. Bile-acid-induced apoptosis involves the activation of (i) the intrinsic (mitochondrial) pathway, triggered by the release into the cytosol of pro-apoptotic mitochondrial factors through pores in the mitochondrial membranes, followed by the activation of executioner caspases, (ii) the extrinsic pathway, triggered by the activation of death receptors localized at the plasma membrane, and (iii) apoptosis by ER (endoplasmic reticulum) stress, involving the activation of executioner caspases without the mediation of mitochondrial factors. UDCA and/or its amide conjugates are instrumental in counteracting all these apoptotic pathways (Figure 2). Importantly, apoptosis by bile acids occurs at concentrations of 25 μM, which can easily be reached in serum in cholestatic diseases [22]. Moreover, apoptosis is prevented by UDCA at low micromolar concentrations [23–25], which can readily be achieved both in serum and liver tissue during therapeutic administration. In line with this, UDCA treatment reduced the amount of apoptotic cells in patients with PBC [26,27]. This anti-apoptotic effect could be indeed therapeutically relevant, since apoptotic bodies are the main source of epitopes involved in autoantigen production in this disease [28].

Intrinsic pathway of apoptosis

This pathway involves, as a triggering event, the bile-acid-induced opening of pores in mitochondrial membranes. This occurs by (i) activation of MPTPs [MPT (mitochondria permeability transition) pores], a non-specific channel formed by proteins from the inner and outer mitochondrial membranes, and (ii) relocalization to mitochondria of pore-forming pro-apoptotic members of the Bcl-2 family of proteins. Generation of both MPT- and Bcl-2-associated pores triggers the activation of executioner caspases through a chain of events initiated by cytochrome *c* release from the intermembraneous space into the cytosol (Figure 2).

UDCA and/or its conjugated derivates can protect against hydrophobic bile-acid-induced impairment of mitochondrial function and integrity in hepatocytes, and probably in cholangiocytes, by inhibiting the most important mitochondrial events leading to apoptosis, i.e. MPT- and Bcl-2-associated pore formation.

UDCA protection against hydrophobic bile-acid-induced MPTP formation Hydrophobic bile acids induce the opening of MPT pores in mitochondria from both rodents [29–31] and humans [32]. MPTP generation results in mitochondrial swelling and uncoupling of oxidative phosphorylation due to the inhibition of respiratory complexes I and III [33]; this leads to decrease in $\Delta\Psi$m (mitochondrial membrane potential) and, eventually, ATP depletion [34].

The mitochondrial perturbation induced by hydrophobic bile acids is also a major source of oxidative stress. Loss of cytochrome *c*, an electron carrier in the respiratory chain, leads to the build up of redox equivalents, with the generation of ROS (reactive oxygen species), mainly superoxide anions and peroxides [29]. Pro-oxidant challenges strongly sensitize hepatocytes to apoptosis by favouring further MPTP formation. Therefore a vicious circle is established in which MPTPs induce ROS generation which, in turn, promotes more MPTP formation. Another source of ROS involves activation of NADPH oxidase, a plasma-membrane enzyme that produces the superoxide radical anion by transferring electrons to O_2 from intracellular NADPH [35]. UDCA protects against all of these pro-oxidant events by (i) preventing MPTP formation and the resulting production of ROS [36,37], and (ii) reinforcing hepatocellular antioxidant defences.

The other antioxidant mechanism of UDCA, i.e. enhancement of antioxidant defences, involves several, complementary mechanisms: (i) UDCA is itself a scavenger of free radicals [38]; (ii) UDCA induces the overexpression of the redox-sensitive transcription factor Nrf2 (nuclear factor-E2-related factor 2), both in mice [39] and in PBC patients [40]; this subsequently increases the levels of the antioxidant enzymes catalase, peroxidase and SOD (superoxide dismutase) [39,41,42]; (iii) UDCA activates the metallothionein IIA promoter [43], increasing the expression of this hydroxyl-radical scavenger [41]; (iv) UDCA increases GSH (reduced glutathione) levels, via the activation of the PI3K (phosphoinositide 3-kinase)/Akt pathway [44].

These protective mechanisms against MPTP formation may contribute not only to protect hepatocytes against apoptosis, but also against necrosis. Apoptosis, an ATP-dependent process, predominates when few mitochondria have been recruited for MPTP formation

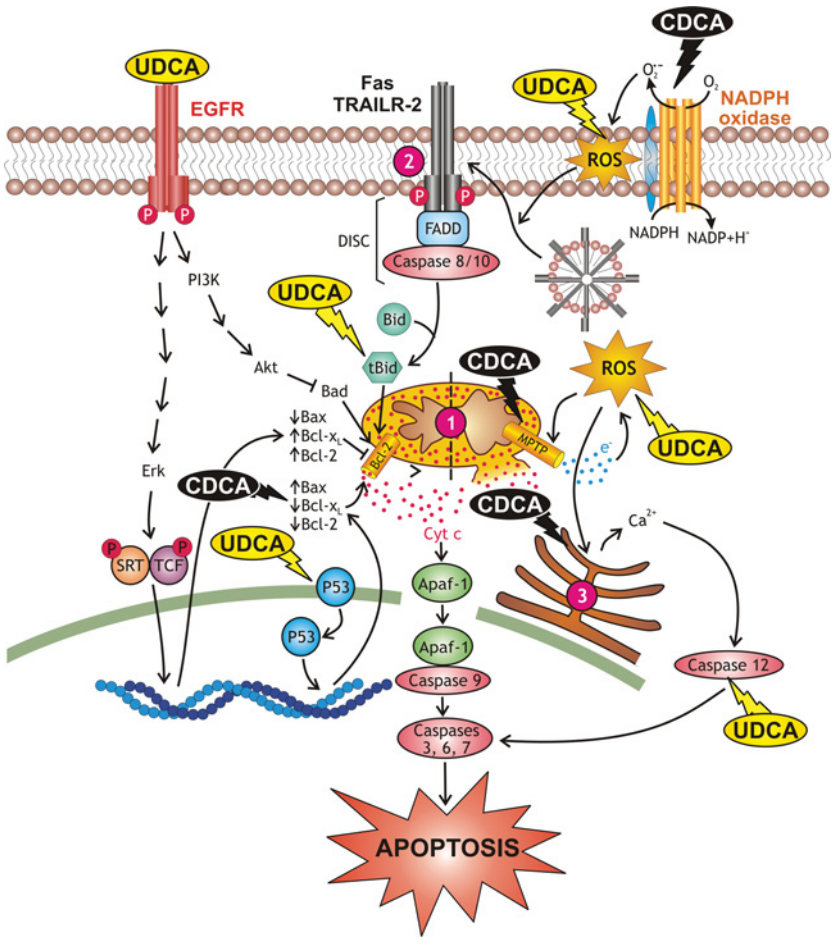

Figure 2 Mechanisms of apoptotic cell death induced by CDCA, the main bile acid retained in cholestasis, and its protection by UDCA

CDCA activates all the three main apoptotic pathways. (1) The intrinsic or mitochondrial pathway, mediated by the release of cytochrome *c* (Cyt *c*) from the mitochondrial intermembrane space into the cytosol. Cytochrome *c* promotes the binding of Apaf-1 (apoptosis protease-activating factor-1) to pro-caspase 9, activation by autocatalysis and, finally, recruitment and activation by caspase 9 of the executioner caspases 3, 6, and 7. Cytochrome *c* release depends on the balance of expression/activity of pore-forming pro-apoptotic proteins of the Bcl-2 family (for example Bax and Bad) and anti-apoptotic proteins (for example Bcl-2 and Bcl-X_L) (see the left-hand side of the mitochondrion), and on the formation of MPTPs facilitated by ROS (see the right-hand side of the mitochondrion). Mitochondrial permeabilization also exacerbates the leakage of electrons from the mitochondrial electron transport chain and further cytosolic ROS generation. (2) The extrinsic pathway, which is initiated by the NADPH-oxidase-mediated ROS-stimulated vesicular trafficking to the plasma membrane of cell death receptors (for example Fas and TRAILR-2), autophosphorylation, association with FADD and pro-caspases 8 and 10 to form the DISC, activation of these caspases, and the subsequent cleavage of Bid to tBid, which promotes mitochondrial Bcl-2-dependent pore formation. (3) Apoptosis by ER stress, which is induced by ROS-mediated Ca^{2+} elevation and executed by caspase 12. Irrespective of the pathways involved, apoptosis proceeds by the downstream action of the executioner caspases 3, 6 and 7. UDCA prevents the activation of all three pro-apoptotic pathways by different mechanisms, namely (i) preventing mitochondrial permeabilization, (ii) inhibiting ROS generation, (iii) counteracting the increased expression of pro-apoptotic mitochondrial proteins (via down-regulation of the transcriptional regulator p53), and (iv) preventing activation of caspase 12. In addition, UDCA stimulates the anti-apoptotic signalling pathways ERK/SRT/TCF and PI3K/Akt by binding to the EGFR. ERK activates by phosphorylation SRF/TCF, a transcription factor that up-regulates the expression of mitochondrial anti-apoptotic proteins, whereas PI3K (via Akt) inhibits the pro-apoptotic protein Bad. See text for more details.

and ATP levels are maintained. On the contrary, necrosis is passive, and therefore predominates when more mitochondria are affected and ATP levels fall [45].

UDCA modulation of mitochondrial Bcl-2 family of proteins Rats fed with the hydrophobic bile acid deoxycholic acid [37] or primary rat hepatocytes exposed to this bile acid [46] had an increase in the mitochondrial content of the Bcl-2 pro-apoptotic protein Bax, and co-administration of UDCA almost fully inhibited this increase.

The effect of Bax is complemented by other members of the Bcl-2 family with either pro-apoptotic or anti-apoptotic properties. Pro-apoptotic Bcl-2 members (for

example Bad, Bax and Bak) homo-oligomerize to form a pore in the outer mitochondrial membrane, whereas the anti-apoptototic ones (for example Bcl-2 and Bcl-X_L) antagonize the function of the pro-apoptotic Bcl-2 proteins either by preventing their mitochondrial translocation (for example Bad) or by sequestering them in mitochondria so as to impede their homo-oligomerization (for example Bax) [47].

The expression of these pro- and anti-apoptotic proteins is regulated by AP-1 (activator protein-1), a dimer of transcription factors formed by one member of the Jun family (c-Jun, JunB and JunD) and one member of the Fos family (c-Fos, FosB, Fra-1 and Fra-2) [48]. The hydrophobic bile acids glycochenodeoxycholic acid [49] and TLCA (taurolithocholic acid) [50] both up-regulate the expression of cFos and JunB, and increase the transcriptional activity of these proteins in Ntcp (Na^+/taurocholic acid co-transporting polypeptide)-transfected HepG2 hepatoma cells. These pro-apoptotic effects are counteracted by TUDCA (tauroursodeoxycholic acid) [50].

UDCA also promotes the activation of intracellular signalling pathways that regulate the expression and function of Bcl-2 proteins. For example, by binding to the EGFR (epidermal growth factor receptor), both UDCA and TUDCA activate anti-apoptotic signalling pathways dependent on the sequential activation of PI3K and the MAPK (mitogen-activated protein kinase) ERK1/2 (extracellular-signal regulated-kinase 1/2) [23,51]. ERK1/2 activates the anti-apoptotic transcription factors SRF (serum-response factor) and TCF (T-cell factor), which act in concert to overexpress anti-apoptotic proteins (Bcl-2 and Bcl-X_L) and to repress pro-apoptotic ones (Bax) [52]. In addition, PI3K also turns on Akt, a downstream protein kinase that phosphorylates and inactivates Bad [53]. The transcriptional effects of UDCA on Bcl-2 proteins are in part due to its ability to inhibit p53, a pro-apoptotic transcription factor that induces Bax and represses Bcl-2 and Bcl-X_L expression. UDCA exerts this effect by halting p53 translocation from the cytosol into the cell nucleus, and by decreasing p53 DNA-binding activity [54]. Finally, UDCA reduces the half-life of p53 by promoting its proteasomal degradation [55].

The anti-apoptotic effect of UDCA is not absolute. Unconjugated UDCA has pro-apoptotic effects on hepatocellular carcinoma cells [56], a factor that might be involved in the clinical observation that UDCA protects cirrhotic patients from hepatocellular carcinoma [57]. Similarly, when normal hepatocytes are exposed for longer periods of time or at increasing concentrations, unconjugated UDCA may exacerbate hydrophobic bile-acid-induced apoptosis *in vitro* [58] and to activate pro-apoptotic mechanisms, such as ceramide formation [58] and release of Ca^{2+} from intracellular stores [59]. Of note, some of these cytotoxic effects were not observed for amide-conjugated UDCA. Since UDCA is efficiently conjugated by the liver with taurine and glycine, even in patients with mild cholestatic liver disease [11,60], studies *in vitro* using unconjugated UDCA may not properly reflect the actual condition *in vivo*. The situation may be, however, different in patients on high UDCA doses or with severe disease. Serum taurine levels are lower [61,62] and biliary excretion of unconjugated bile acids is higher [63] in untreated cirrhotic patients, suggesting defective conjugation. If so, this may be a contributing factor to the unfavourable clinical outcome in patients taking high UDCA doses (28–30 $mg \cdot kg^{-1}$ of body weight $\cdot day^{-1}$) to treat either PSC [13] or NASH (non-alcoholic steatohepatitis) [64,65], since apoptosis plays a critical role in these hepatopathies [66,67].

Extrinsic pathway of apoptosis

This pathway is initiated by bile-acid-activated vesicular trafficking from Golgi to the plasma membrane of death receptors, such as Fas [68] and TRAILR-2 [TNF (tumour-necrosis-factor)-related apoptosis-inducing ligand receptor-2) [69]. This is followed by spontaneous homo-oligomerization of these receptors, formation of the DISC (death-inducing signalling complex) by association with the adaptor molecule FADD (Fas-associated death domain) and pro-caspases 8 and 10, and finally, activation of these caspases (Figure 2). Bile-acid-induced Fas activation in hepatocytes occurs in a FasL (Fas ligand)-independent manner, through a mechanism that involves NADPH oxidase-dependent ROS generation in the plasma-membrane environment [35].

In hepatocytes, progression of the apoptotic cascade after caspase 8/10 activation depends on its amplification by mitochondria [70]. These caspases produce proteolysis of Bid, and its truncated form, tBid, translocates to mitochondria. Once there, tBid induces conformational changes in pro-apoptotic Bax and Bak, which trigger their homo-oligomerization and pore formation.

UDCA inhibits the extrinsic pathways mediated by Fas, probably by interfering with the action of tBid on mitochondria [71], and perhaps by counteracting ROS-induced Fas activation, due to its antioxidant properties as mentioned above.

Apoptosis by ER stress

ER stress occurs when protein production and trafficking systems in the organelle break down, leading to accumulation of misfolded proteins and, eventually, to apoptosis. This is triggered by factors such as oxidative stress, ER Ca^{2+} accumulation, nutrient deprivation and a number of toxic insults, among others [72].

This pro-apoptotic pathway is not fully understood, but both disruption of Ca^{2+} homoeostasis and caspase 12 activation are pivotal events [73,74]. Hydrophobic bile acids can induce apoptosis by this mechanism

by generating ROS [75]. ROS triggers Ca^{2+} release from the ER, with the subsequent activation of caspase 12, which in turn activates executioner caspases. This is mediated by the pro-apoptotic proteins Bax and Bak, which are also localized at the ER membrane, and regulate the Ca^{2+} channel inositol 1,4,5-trisphosphate receptor. Ca^{2+} release from the ER can activate calpains, which in turn proteolytically activate caspase 12 to mediate apoptosis. In addition, misfolded proteins can activate the pro-apoptotic JNK (c-Jun N-terminal kinase)-mediated signalling pathway and induce the expression of the pro-apoptotic transcription factor CHOP (CCAAT/enhancer-binding protein-homologous protein), which modulates activity and expression of Bcl-2 family proteins [72].

UDCA may halt these pro-apoptotic mechanisms at different levels. UDCA prevents caspase 12 activation [76], the generation of oxidative stress, and the alterations in the balance between pro- and anti-apoptotic Bcl-2 proteins (see above). In addition, TUDCA acts as a chemical chaperone that reduces ER stress [77].

Apoptosis by ER stress occurs in several liver diseases, including NASH and viral hepatitis [72]. This perhaps contributes to explain the alleged beneficial effects of UDCA as a co-adjuvant therapeutic agent in NASH. Indeed, preliminary studies have shown that a combination of vitamin E and UDCA improved liver histology, hepatocellular apoptosis and circulating adiponectin levels in these patients [78]. On the other hand, the anti-apoptotic effects of UDCA may be detrimental in viral hepatitis, since apoptosis was suggested to be a mechanism for viral elimination [79]; perhaps this contributes to the inability of the drug to enhance viral clearance [80]. ER stress has also been associated with the pathogenesis of many non-hepatic diseases, such as diabetes, atherosclerosis and neurodegenerative diseases [81,82]. This has prompted the experimental therapeutic assay of UDCA and its conjugates in animal models of these diseases, with encouraging results so far [77,83–86].

Effect of UDCA on apoptosis induced by other mediators of cholestatic disease

UDCA inhibits apoptosis induced by compounds other than bile acids. They include the pro-inflammatory cytokines TGF-β1 (transforming growth factor-β1) [87], TNF-α [88] and FasL [71]. These cytokines activate death receptors critically involved in both cholangiolar and hepatocellular apoptosis in chronic cholestatic disease [89]. Apoptosis is critically involved in bile duct loss [90], and apoptotic debris contributes to activation of collagen-producing hepatic stellate cells [91]. Therefore the protective effects of UDCA in both bile duct loss and fibrogenesis, two common features in most chronic cholestatic hepatopathies, may involve the prevention of cytokine-induced apoptosis.

For TGF-β1 at least, the anti-apoptotic effect of UDCA requires binding of UDCA to the glucocorticoid receptor and the further translocation of this receptor to the nucleus [92]. Binding of UDCA to the glucocorticoid receptor also suppresses the transcriptional activity of NF-κB (nuclear factor κB), whose expression is induced by pro-apoptotic inflammatory cytokines [93]. NF-κB inhibition may, however, be regarded as unbeneficial, as it induces the expression of anti-apoptotic genes as well, and its activation in cholestasis was suggested to confer resistant against apoptosis [94]. Irrespective of this dual action in apoptosis, the ability of UDCA to bind and activate the glucocorticoid receptor renders the drug a pleiotropic agent playing an important role as an anti-inflammatory agent (see below).

Activation of TRAILR-2, the receptor for TRAIL, is a major mechanism in cell death and mediates cholestatic liver injury [95]. Healthy cholangiocytes do not express TRAIL, but it is up-regulated in cholangiocytes in PBC and PSC, and this may activate the TRAIL/TRAILR-2 pro-apoptotic pathway [95]. Whether UCDA halts this cholangiodestructive mechanism remains to be ascertained.

These anti-apoptotic properties of UDCA may help to explain the widespread use of this drug with variable success in non-cholestatic liver diseases, in which these cytokines play a crucial role, such as alcoholic and non-alcoholic steatohepatitis [78,96], hepatitis induced by drugs (for example methotrexate [97] and amoxicillin/clavulanic acid [98]) and cholestatic viral hepatitis C [99–101]. Furthermore, TUDCA has been shown to have neuroprotective effects in animal models of neurological disorders with deregulation of apoptosis, such as haemorrhagic stroke [101], and Alzheimer's [84], Parkinson's [85] and Huntington's [86] neurodegenerative diseases, and to inhibit apoptosis induced by different stimuli in isolated neuronal cells [102,103]. Clinical studies are awaited to confirm these benefits in patients.

Effect of UDCA on cytotoxic bile-acid-induced hepatocellular oncotic necrosis

Oncotic necrosis is characterized by cell swelling and disruption of plasma-membrane integrity, with the release of cytosolic proteins into blood [for example LDH (lactate dehydrogenase) and transaminases] as a characteristic event. Necrosis is a major mechanism of cell death in animal models of cholestasis [104–106] and in cholestatic human diseases, such as PBC [107,108], PSC [109] and PFIC-2 [110]. Protection by amide-conjugated UDCA against hydrophobic bile-acid-induced oncotic necrosis has been shown both in isolated hepatocytes [19,111–113] and in rats *in vivo* [114]. This

may have a clinical correlate, since hepatocellular necrosis is attenuated by UDCA treatment in PBC patients [108].

Hydrophobic bile acids can induce oncotic necrosis by two main mechanisms, namely oxidative-stress-induced lipid peroxidation [29,30,115–117] and solubilization of the hepatocellular plasma membrane [118,119], and both of these can be counteracted by UDCA.

Protection by UDCA against necrosis resulting from bile-acid-induced oxidation

As stated above, bile acids induce ROS formation from a mitochondrial origin by inducing MPTPs. Exposure to high levels of hydrophobic bile acids eventually leads to ATP depletion [34], a factor that shifts cell death from apoptosis to necrosis [45]. In addition, the associated lipid peroxidation causes plasma-membrane breakdown, with the subsequent escape of cytosolic proteins (for example LDH and transaminases), a hallmark of the necrosis process. The importance of oxidative stress is supported by studies showing that antioxidants either totally [115] or partially [118] prevent this process, depending on the bile acid concentration.

The protective effects of UDCA against bile-acid-induced oxidative stress discussed above may explain in part the protection of UDCA on the detrimental oxidative effects of toxic bile acids. However, the actual importance of this UDCA-protective mechanism remains to be established with certainty.

Protection by UDCA against bile-acid-induced membrane solubilization

At high bile acid concentrations, ROS scavengers can completely prevent lipid peroxidation without fully blocking bile-acid-induced cytolysis [118]. Furthermore, a recent study has shown that antioxidants do not attenuate this process at all in rat hepatocytes in primary culture [120]. This suggests that mechanisms other than oxidative stress are involved. As an additional mechanism, detergent bile acids have been proposed to exert direct solubilization of the hepatocellular plasma membrane and further necrosis by cytolysis [19,111,112,118].

A direct stabilization of the plasma membrane by UDCA against tensioactive bile acids has been suggested from studies in artificial membranes (liposomes) [121–123]. This finding was recently reproduced in isolated hepatocyte plasma membranes, where TUDCA prevented the bilayer-to-micelle transition induced by CDCA [113]. This membrane-stabilizing effect seems to involve the formation of a complex between cholesterol and the anionic form of TUDCA occurring at physiological pH, which favours electrostatic repulsion of negatively charged tensioactive bile acids [123,124]. It has been also suggested that TUDCA interferes in bulk solution with the formation of mixed micelles composed of membrane lipids and detergent bile acids [125]. However, lipid membranes previously exposed to TUDCA remain refractory to the solubilizing effect of bile acids irrespective of whether TUDCA is further present or not in the incubation medium [113].

A limitation of these findings is that millimolar concentrations of conjugated UDCA are required to exert membrane-stabilizing effects, but only micromolar levels are reached in systemic circulation under normal therapeutic conditions. Millimolar concentrations of conjugated UDCA can, however, occur in the biliary lumen, and UDCA conjugates may exert protective effects from there, where endogenous bile acids also reach cytotoxic concentrations. This protective mechanism may particularly contribute to the beneficial effect of UDCA treatment in hepatopathies involving genetic defects in the canalicular phospholipid translocator MDR3 (multidrug-resistance protein 3 {ABC4B4 [ABC (ATP-binding cassette) sub-family B member 4]}), such as PFIC-3 or the so-called 'anti-mitochondrial antibody-negative PBC' [126]. In these hepatopathies, phospholipid deficits in bile enhances bile acid cytolytic effects, as it increases the amount of bile acid monomers in bile, which otherwise would form mixed micelles with biliary phospholipids and cholesterol.

Protection by UDCA against impairment of cholangiocyte viability

As with hepatocytes, cholangiocytes are highly exposed to cytotoxic bile acid levels, as they are also in direct contact with bile. However, the ultrastructural integrity of isolated rat perfused livers exposed to unconjugated bile acids remain intact, even when livers from rats with impaired bile acid conjugation to increase bile acid toxicity were assayed [127]. On the contrary, hepatocytes showed a prominent subcellular damage [127]. This difference was attributed to the active extrusion of bile acids from the cholangiocyte and the cytoprotective presence of phospholipids in bile. Therefore the (T)UDCA survival mechanisms against bile-acid-induced cell death discussed above for hepatocytes may not necessarily apply to cholangiocytes. Nevertheless, bile-duct loss by apoptosis in cholangiopathies such as PBC and PSC, which have normal phospholipid contents in bile, is mainly immune- rather than bile-acid-mediated [128]. In these cases, the anti-apoptotic properties of UDCA against cytokines (see above), as well as immunomodulatory properties of UDCA (see below), may play a key role. Interestingly, in human cholangiocytes in culture glycoconjugated UDCA prevented both the cytochrome *c* release and caspase 3 activation induced by beauvericin [129], a compound that shares common pro-apoptotic mechanisms with cytokines. In addition, UDCA stimulates the biliary detoxification of NO as *S*-nitrosoglutathione, and the latter compound protects cultured rat cholangiocytes

from apoptosis by activation of the PI3K/Akt survival pathway [130]. UDCA also decreased both nuclear DNA fragmentation and pro-apoptotic protein Bcl-2 levels in biliary epithelial cells from PBC patients [27]; this suggests that the anti-apoptotic effects of UDCA at the cholangiocyte level could be of actual clinical relevance.

Modulation of the expression of liver transporters and enzyme systems by UDCA

Effects of UDCA on hepatocellular bile acid transporters and metabolizing enzymes under normal and cholestatic conditions

UDCA exerts part of its therapeutic effects by reducing the intracellular content/cytotoxicity of bile acids and other potentially toxic cholephilic compounds accumulated by the secretory failure. At the same time, it favours the elimination of these compounds via alternative routes, mainly the urinary route. This is achieved by: (i) hindering basolateral bile acid uptake, (ii) overexpressing basolateral export pumps, and (iii) repressing bile acid synthesis and favouring bile acid hydroxylation.

Most of these beneficial changes are due to the ability that UDCA has to induce these changes in normal livers, i.e. without a cholestatic context (Figure 3).

UDCA feeding to normal mice down-regulates Oatp1 {organic anion-transporting polypeptide 1 [Slco1 (solute carrier organic anion transporter family member 1)]}, a basolateral transporter engaged in bile acid hepatocyte uptake, by a transcriptional mechanism [131]. The expression of the other major basolateral bile acid uptake system Ntcp [Slc10a1 (solute carrier family 10 member 1)] remains unaltered [131]. In addition, UDCA administration up-regulates the basolateral export pumps Mrp3 {multidrug resistance-associated protein 3 [Abcc3 (ABC sub-family C member 3]} [132] and Mrp4 [Abcc4 (ABC sub-family C member 4)] [133], both at mRNA and protein levels. This facilitates the spillover into blood of amidated and sulfated bile acids (mainly via Mrp4) and glucuronides of bilirubin and bile acids (mainly via Mrp3), which favours their further urinary excretion. Overexpression of these basolateral export pumps explains the increased serum content and urinary excretion of sulfated and glucuronidated bile acids, particularly those of UDCA, both in normal subjects [134] and in patients with PBC on UDCA treatment [135]. This is explained further by the more efficient UDCA glucuronidation [136] and sulfation [137] compared with most bile acids, and perhaps by the improvement in the intrinsic transport capability of the kidney to excrete these compounds. Indeed, UDCA feeding to mice stimulated the expression of the renal apical bile acid export pumps Mrp2 [132] and Mrp4 [133], and repressed the expression of Asbt {apical sodium-dependent bile salt transporter [Slc10a2 (solute carrier family 10 member 2)]}, a transporter involved in the tubular reabsorption of bile acids [133].

Feeding of UDCA to rodents also up-regulates hepatocellular canalicular export pumps [131]. They comprise Bsep {bile salt export pump [Abcb11 (ABC sub-family B member 11)]}, the main canalicular transporter of amide-conjugated bile acids, and Mrp2 [Abcc2 (ABC sub-family C member 2)], which excretes into bile glucuronidated/sulfated bile acids, GSH and GSSG (oxidized glutathione), and many other negatively charged xeno- and endo-biotics, including bilirubin glucuronides. As bile acids and GSH/GSSG are the main osmotic driving forces for bile flow generation, the up-regulation of both Bsep and Mrp2 would contribute to the choleretic effect of UDCA.

In addition to transporters, UDCA also modulates the expression in the liver of enzymes involved in bile acid metabolism (Figure 3). UDCA administration represses in rodents the expression of the cytochrome P450 enzyme Cyp7a1 (cytochrome P450 family 7 subfamily A polypeptide 1), which mediates the rate-limiting step of the classic bile-acid-biosynthetic pathway [133]. In addition, it stimulates in mice the sterol hydroxylases responsible for bile acid hydroxylation, such as Cyp3a11 (cytochrome P450 family 3 subfamily a polypeptide 11) and Cyp2b10 (cytochrome P450 family 2 subfamily b polypeptide 10) [133,138], as well as CYP3A4 (cytochrome P450 family 3 subfamily A polypeptide 4) in primary cultured human hepatocytes [138]. These metabolic changes decrease both the endogenous production of bile acids and the potentially deleterious toxicity of the remaining ones, as hydroxylation of bile acids decreases their hydrophobicity.

The transcriptional regulation by UDCA of both hepatic transporters and enzymatic systems seems to involve, in part, its role as an activating ligand of the nuclear receptors PXR (pregnane X receptor) and FXR (farnesoid X receptor). These nuclear receptors have complementary roles in the regulation of genes involved in lipid and lipoprotein metabolism, including bile acid synthesis and transport, and are actively involved in the spontaneous adaptive response in cholestasis triggers by accumulated bile acids, aimed at reducing the synthesis and increasing the extrahepatic disposal of these potentially toxic compounds (for reviews, see [139,140]). By sharing with endogenous bile acids the ability to bind to these nuclear receptors, UDCA would reinforce this adaptive response without adding toxicity.

UDCA is a relatively strong PXR agonist, but a weak FXR agonist [138]. Therefore a major role for FXR in the transcriptional effects of UDCA is unlikely. This is in line with studies of UDCA-fed FXR-knockdown mice, where similar changes to wild-type were observed in the expression of ABC transporters in the liver

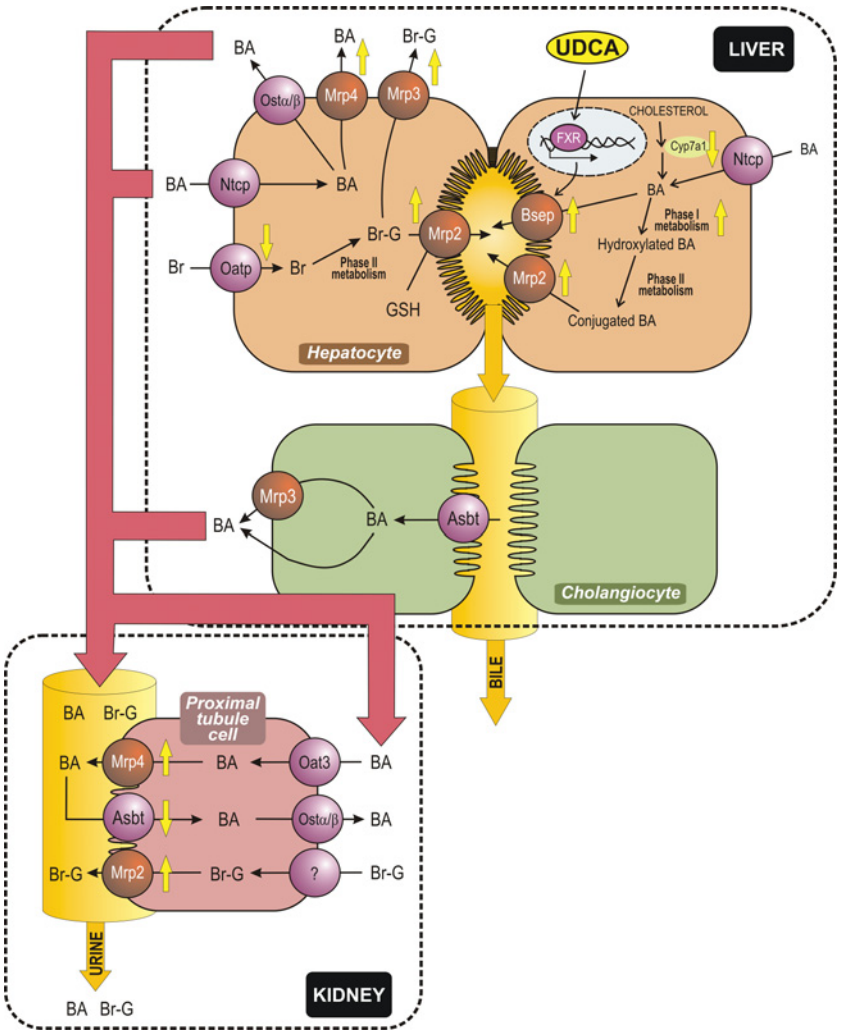

Figure 3 Effects of the administration of UDCA to rodents on the expression of transporters and enzymes involved in bile acid and bilirubin detoxification

UDCA stimulates the expression and/or activity of the main canalicular transporters involved in bile formation (Bsep and Mrp2). For Bsep at least, this involves the transcription factor FXR. UDCA also favours the reflux back to plasma of potentially toxic endogenous compounds accumulated over the cholestatic process, such as BAs (bile acids) and Br-G [glucuronized Br (bilirubin)]. This involves the down-regulation of export pumps in the sinusoidal membrane (Mrp3 and Mrp4) and the down-regulation of transporters involved in BA/Br uptake (Oatp). Such a reflux redirects these toxic metabolites to the kidney for depuration via glomerular filtration and tubular renal excretion, the latter process being facilitated by the up-regulation of apical kidney transporters (Mrp2 and Mrp4). Finally, UDCA represses the hepatic synthesis of endogenous BAs by down-regulating Cyp7a1, the cytochrome P450 that mediates the rate-limiting step in BA biosynthesis, and facilitates BA hydroxylation, rendering lower endogenous levels of BAs with reduced toxicity. See the text for more details.

(Mrp3, Mrp4 and Mrp2), kidney (Mrp2 and Mrp4) and gut (Mrp2) [132,133]. The only exception was Bsep, which was not up-regulated in FXR-knockdown animals [132,133]. Essentially, the same induction pattern and a similar lack of FXR-dependency was observed both in rats fed on cholic acid [132,133] and in bile-duct-ligated animals [141], which suggests that endogenous bile acids accumulated by the cholestatic failure shares similar induction mechanisms to UDCA. This may explain the limited protective response in terms of transporter induction afforded by UDCA administration to bile-duct-ligated mice [142].

The predominant FXR-independent response to UDCA suggests the participation of other nuclear receptors in the transcriptional effects of UDCA. The high affinity of UDCA for PXR makes this nuclear receptor a likely candidate [143]. However, studies in PXR-knockdown animals are unfortunately lacking.

These results in laboratory animals should, however, be extrapolated to humans with caution. Only some of the effects of UDCA on the expression of transporters/enzymes observed in rodents, or even in human cell lines *in vitro*, have been reproduced in humans. Otherwise healthy patients who received

UDCA for gallstone dissolution had increased levels of BSEP and MRP4, but not of MRP2, MRP3 and OATP1 [144]. The bile-acid-metabolizing enzymes CYP3A4 (hydroxylation) and UGT1A1 (UDP glucuronosyltransferase 1 family polypeptide A1) (glucuronidation) also remained unchanged [144]. Surprisingly, the limited potential of UDCA to induce transcriptional changes in humans is complemented by the classical PXR ligand rifampicin, despite the alleged role for UDCA as a strong PXR ligand. Indeed, unlike UDCA, rifampicin enhances the expression of CYP3A4, UGT1A1 and MRP2 [144], and improves the CYP3A4-dependent metabolism of budesonide and cortisol [145]. These results would justify the combined use of UDCA and rifampicin for the treatment of cholestasis in UDCA-non-responsive patients.

Although many transporter/enzymes are not regulated by UDCA administration to healthy individuals, the situation may be different in cholestatic patients, whose expression of these proteins is pathologically altered as part of the course of the disease. For example, unlike healthy individuals [144], patients with early-stage PBC, who have a slightly decreased expression of MRP2, showed an improvement in the levels of these transporter after UDCA treatment [146]. This more probably reflects the ability of UDCA to counteract cholestatic factors involved in the pathobiology of the disease rather than its capability to induce Mrp2 expression. In line with this, UDCA only improved the decreased expression of MRP2 in early-stage PBC, when cholestatic effects of pro-inflammatory cytokines are the predominant causal factor [147]. If the anti-cholestatic properties of UDCA involve counteraction of the effects of cytokines, this may also apply to, and explain, therapeutic effects of UDCA in other inflammatory cholestatic disease, such as parenteral-nutrition-induced cholestasis [148,149] and cholestatic hepatitis of viral [150] or toxic [151,152] origin. More information on the ability of UDCA to improve transporter function and expression in inflammatory cholestatic disease is warranted.

The potential ability of UDCA to up-regulate the canalicular export pumps Mrp2 and Bsep in a cholestatic context may be relevant in explaining the protective mechanism of this bile acid in cholestatic diseases of both a non-obstructive and non-inflammatory nature in which impaired expression of these transporters is a primary causal factor. This is the situation in hereditary cholestatic diseases with partial transporter defects [153], or functional impairment of transporter activity by drugs [154] or sex hormones, as in pregnancy-induced cholestasis [10,155]. In the latter pathology, susceptible women have low basal constitutive levels of these transporters, in many cases because of genetic mutations [156]. To this basal impairment, pregnancy adds the effects of cholestatic hormones that build up under this condition (oestrogens and progesterone), which results in an overt cholestatic phenotype. The cholestatic oestrogen 17α-ethinyloestradiol, administered to experimental animals to mimic oestrogen-associated cholestatic disease in patients, impairs the constitutive expression of both Bsep [157,158] and Mrp2 [156,159]. In this model, UDCA prevents the associated functional failure of these transporters by enhancing carrier constitutive expression (as for Bsep) [157] or transporter activity without restoring protein level (as for Mrp2) [159]; the latter effect may involve a positive interaction with either a putative regulatory site of the transporter or its lipid microenvironment. These beneficial effects of UDCA are, in part, due to the ability of UDCA and its taurine conjugate to partially inhibit the UGT2B1 (UDP glucuronosyltransferase 2 family polypeptide B1)-mediated 17β-glucuronidation of 17α-ethinyloestradiol, which is a prerequisite for its cholestatic action [160].

Unlike what happens in hepatocellular cholestasis, the induction that UDCA administration exerts at the level of expression/function of canalicular transporters may be deleterious in processes where obstructive cholestasis is the predominant feature, for example in PSC with bile duct strictures, or other late-stage 'vanishing' bile-duct syndromes, such as PBC or biliary atresia. Under these conditions, an improvement in bile flow generation may aggravate biliary infarcts above the obstruction, as has been shown in bile-duct-ligated mice [143]. This could partly explain the limited therapeutic effects of UDCA in late-stage PBC [161] and in patients with PSC [13], as well as its selective therapeutic efficacy in biliary atresia, restricted to the type III classification (ductules >50 μm) [162].

Modulation of cholangiocyte transport and ductular bile flow by UDCA

Cholangiocyte dysfunction is a common event in many chronic cholestatic diseases and it may play a role in the progression of the disease. Lack of HCO_3^--rich bile secreted at the ductular level may concentrate potentially toxic biliary constituents, for example detergent bile acids, and thus contribute to cholangiocyte damage. The distinctive property of UDCA to stimulate bile flow in excess of what is expected from its osmotic properties (hypercholeresis) may contribute to dilute these toxic biliary solutes [163]. Indeed, UDCA stimulates HCO_3^- secretion both in rats [164] and in humans [165]. This is due to its distinctive ability to both undergo cholehepatic shunting and directly stimulate cholangiocyte secretion (see Figure 4 for further details).

Acting through this mechanism, UDCA is thought to reduce portal inflammation, ductular proliferation and fibrosis in mice lacking the canalicular phospholipid translocator Mdr2 (Abcb2), an experimental model for sclerosing cholangitis [166]. This ameliorating effect is, however, only observed in early stages of the cholangiopathy, but not when regional fibro-obliteration

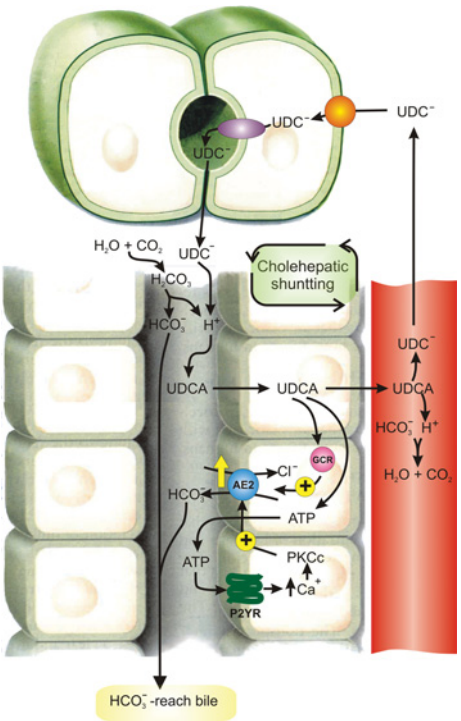

Figure 4 Mechanisms of UDCA-induced HCO_3^--rich hypercholeresis

UDCA stimulates HCO_3^- secretion and concomitant ductular bile flow formation both by undergoing cholehepatic shunting and by directly stimulating cholangiocyte secretion. UDCA cholehepatic shunting involves the passive absorption of the bile acid in its protonated (uncharged) form by the cholangiocyte, followed by its transfer back to the hepatic sinusoids via the peribiliary plexus, to be returned to cholangiocytes by hepatocyte resecretion into bile, as ursodeoxycholate (UDC^-). UDC^- protonation renders a HCO_3^- molecule in the lumen each time UDCA suffers a cycling event, which acts as a osmotic driving force for ductular bile formation. In addition, UDCA activates AE2, the main transporter involved in ductular HCO_3^- secretion, via both transcriptional and post-transcriptional signalling mechanisms. The latter process involves stimulation of ATP release by both hepatocytes and cholangiocytes into bile, which facilitates ATP-mediated activation of purinergic 2Y receptors (P2YR). These receptors stimulate AE2 through an increase in free cytosolic Ca^{2+} and the further activation of cPKC (PKCc) isoforms, which in turn activate a variety of Cl^- channels present in the apical cholangiocyte domain required for AE2-mediated Cl^-/HCO_3^- exchange. See the text for more details.

of bile ducts becomes apparent [143]. The C_{23} homologue of UDCA 24-norUDCA, which has one less methyl group in its side chain, is even more efficient than UDCA in generating HCO_3^--rich hypercholeresis and in preserving the bile duct integrity in Mdr2-knockout mice [167]. The superior performance of norUDCA may be due to (i) its even lower amide conjugation than UDCA, which further favours passive reabsorption, and (ii) its greater osmotic effect, since it is mostly present in a monomeric rather than in a micellar form [168]. Ductular hypercholeresis, together with the enrichment of UDCA in bile at the expense of more toxic bile acids (see above), may help to explain the improvement in serum liver tests in 60 % of patients with PFIC-3 lacking the canalicular phospholipid translocator MDR3 [169]; lack of biliary phospholipids renders detergent bile acid more toxic against cholangiocytes [170].

The impairment of ductular bile flow in cholestatic cholangiopathies is multifactorial. The ductular secretion of HCO_3^- is reduced in PBC [171] and cystic fibrosis [172]. This is due to alterations in either or both the expression and function of transport systems involved in ductular HCO_3^- excretion, namely AE2 (anion exchanger 2) for PBC [173], and the Cl^- channel CFTR (cystic fibrosis transmembrane conductance regulator) for cystic fibrosis [172]. The transcriptional expression of the Cl^-/HCO_3^- counter-transporter AE2 [SLC4A2 (solute carrier family 4 anion exchanger member 2)], the main transporter responsible for secretin-stimulated ductular bile flow, is decreased in PBC patients and improved by UDCA treatment [173]. Furthermore, treatment with UDCA plus dexamethasone, an alternative therapeutic combination for PBC patients with an incomplete response to UDCA monotherapy, up-regulated AE2 and enhanced its anion exchange activity in human liver cells from both cholangiocyte and hepatocyte lineages [174]. This occurs via the binding of both UDCA and dexamethasone to the glucocorticoid receptor, which interacts with liver-enriched HNF-1 (hepatocyte nuclear factor-1) to enhance the transcriptional activity of the AE2 alternate promoter [174]. UDCA also activates AE2 function via post-transcriptional signalling mechanisms. It stimulates the ATP release by hepatocytes [175] and cholangiocytes [176] into bile, and luminal ATP activates purinergic 2Y receptors. These receptors stimulate AE2 through an increase in free cytosolic Ca^{2+} and the further activation of cPKC (Ca^{2+}-dependent protein kinase C) isoforms, which activate a variety of Cl^- channels present in the apical cholangiocyte domain required for Cl^-/HCO_3^- exchange [176]. UDCA-mediated activation of these kinases also reduces cholangiocyte proliferation and ductular secretion in bile-duct-ligated rats, an effect mediated by inhibition of the uptake of mitogenic hydrophobic bile acids via Asbt down-regulation [177].

UDCA-induced normalization of the altered cellular localization of hepatocellular transporters in cholestasis

Endocytosis into subapical vesicular compartments of Bsep and Mrp2, the two canalicular export pumps involved in bile flow generation, has been described in several experimental models of cholestasis, including cholestasis induced by $E_2$17G (oestradiol 17β-glucuronide) [178,179], TLCA [180] and bile-duct ligation [181].

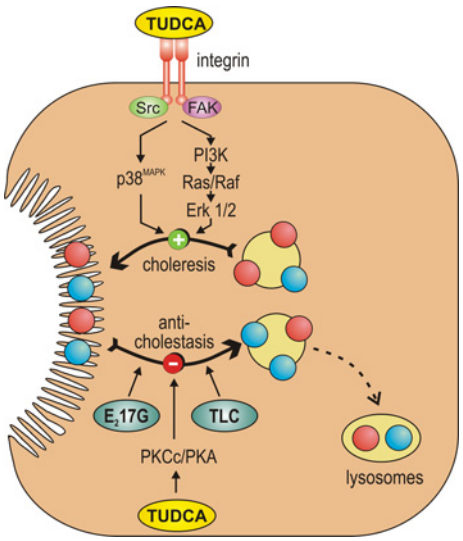

Figure 5 Choleretic and anti-cholestatic mechanisms of the taurine-conjugated metabolite of UDCA (TUDCA) by modulation of the localization status of canalicular transporters

TUDCA induces choleresis by stimulating exocytic insertion into the apical membrane domain of canalicular transporters involved in bile formation. This choleretic mechanism involves integrin-sensing and FAK/Src activation, followed by the dual activation of the MAPKs $p38^{MAPK}$ ERK1/2. ERK1/2 activation requires the upstream sequential activation of PI3K and Ras/Raf. TUDCA also exerts anti-cholestatic effects against cholestatic drugs that induce endocytic internalization of canalicular transporters as part of their cholestatic mechanisms, such as $E_2$17G or TLCA. Unlike choleresis, this anti-cholestatic effect seems to involve co-activation of cPKC and PKA. See the text for more details.

This internalization is associated with a decrease in bile flow and an impairment in the biliary output of cholephilic compounds, suggesting that this phenomenon is a key mechanism triggering cholestasis (Figure 5). A similar phenomenon has been described in different human cholestatic diseases, including (i) obstructive extrahepatic cholestasis [182,183], (ii) pregnancy-induced cholestasis [184], (iii) inflammatory cholestasis associated with autoimmune hepatitis [183], (iv) mixed (obstructive plus inflammatory) cholestatic diseases, including PBC [185] and PSC [183], and (v) cholestasis induced by drugs, such as antibiotics, thiopronine, chlorpromazine and anti-inflammatory drugs [183,186]. Since sustained internalization of these transporters may lead to delivery to the lysosomal compartment followed by degradation, this phenomenon may explain, in part, the decrease in the post-transcriptional expression of transporters frequently observed in these hepatopathies.

TUDCA stimulates the opposite process as part of its choleretic effects, i.e. the exocytic insertion of canalicular transporters. TUDCA activates two MAPK-dependent pathways, $p38^{MAPK}$ [187] and ERK1/2 [188], within minutes, and this effect involves integrin-sensing and FAK (focal adhesion kinase)/Src activation as upstream events [189]. Dual MAPK activation by TUDCA was causally linked to both increased biliary excretion of bile acids and canalicular insertion of Bsep (the latter demonstrated only for $p38^{MAPK}$ [187]). The stimulus induced by TUDCA on ERK1/2, but not on $p38^{MAPK}$, is dependent on the sequential activation of PI3K and Ras/Raf [190]. The two MAPK-dependent pathways seem to act in parallel, and dual activation is required [187]. Studies in human HepG2 cells and in rat hepatocytes in culture showed that TUDCA-stimulated insertion of BSEP/Bsep involves not only increased targeting from the subapical compartment, but also enhanced its trafficking from the Golgi complex to the subapical compartment, and that $p38^{MAPK}$ is a key signalling mediator of this latter effect [191].

By stimulating exocytosis, TUDCA was thought to counteract the endocytic internalization of both Bsep [192] and Mrp2 [193] in TLCA-induced cholestasis in rats. This effect, however, involves a different set of protein kinases from those promoting exocytosis under normal conditions. The anti-cholestatic effects of TUDCA is not mediated by MAPKs [194]; it rather involves cPKC, through a co-operative mechanism with PKA (protein kinase A) [195] (Figure 5). Therefore TUDCA-stimulated transporter exocytosis (under normal conditions) and TUDCA-induced anti-cholestatic effects by prevention of transporter endocytosis may be independent events.

If confirmed in humans, the ability of UDCA metabolites to prevent endocytosis of canalicular transporters may be involved in the improvement in the canalicular transporter expression in the broad range of human cholestatic diseases, where endocytic internalization of canalicular transporters has been shown and where it may be a triggering event.

Immunoregulatory properties of UDCA

Liver immunological attack in cholestatic autoimmune diseases occurs by: (i) the existence of an exacerbated immune response caused by the loss of tolerance to autoantigens, and (ii) aberrant overexpression of antigens of both MHC-I (in hepatocytes) [196] and MHC-II (in cholangiocytes) [197].

UDCA has immunomodulatory effects on both the exacerbated response of immunological cells (immunosuppressive properties), and on the aberrant overexpression of MHCs and intercellular adhesion molecules involved in the immunological attack on the liver occurring in PBC and PSC (Figure 6). Anti-inflammatory effects of UDCA administration in the portal tract may explain, in part, the beneficial effect of UDCA on fibrosis progression, as shown in bile-duct-ligated rats [198] and in patients with PBC [199]. These

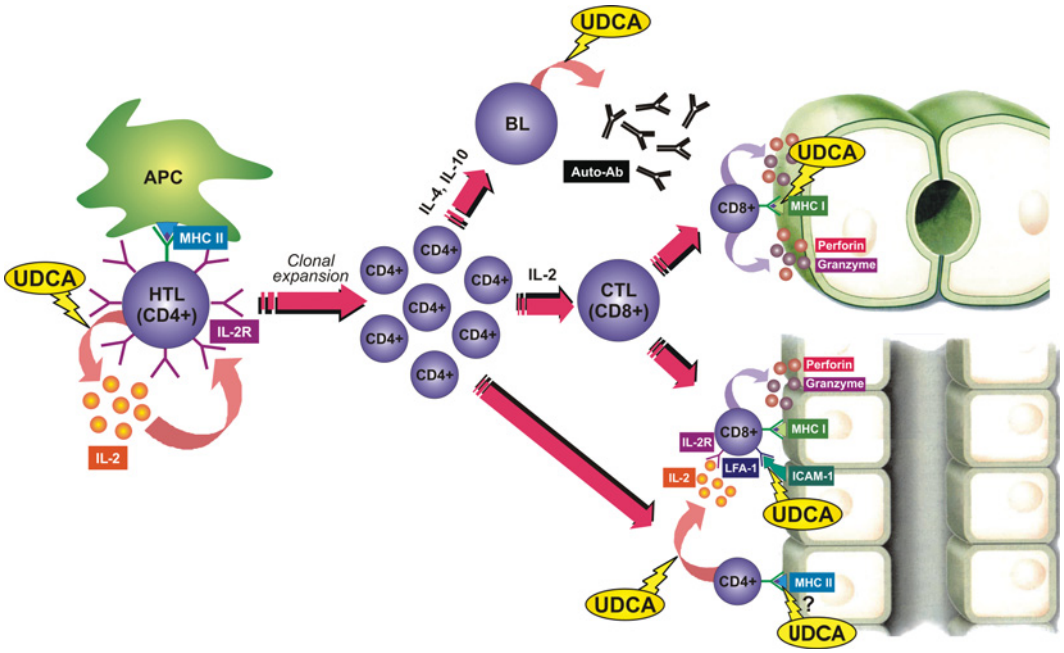

Figure 6 Inhibition by UDCA of the exacerbated immunological response occurring in autoimmune hepatopathies

Autoantigen-presenting cells (APCs) stimulate HTLs ($CD4^+$) to produce and release IL-2, which triggers autocrine activation and clonal proliferation of these cells via binding to its receptor IL-2R. These cells, in turn, activate B-lymphocytes (BLs) for the production of auto-antibodies (humoral response) and CTLs ($CD8^+$) (cellular response). CTLs attack both cholangiocytes and hepatocytes by releasing perforin and granzyme, inducing cellular death by necro-apoptosis. The binding of CTLs to hepatocytes is facilitated by the overexpression of the MHC-I, which is induced by bile acids accumulated over the cholestatic process, mainly CDCA. In cholangiocytes, CDCA induces overexpression of the MHC-II, which locally favours the sequential activation of HTLs and CTLs, leading to cholangiocyte death. This is reinforced by the overexpression of both ICAM-1 and LFA-1 in cholangiocytes and lymphocytes respectively. UDCA counteracts the production of auto-antibodies by BLs and inhibits the production of IL-2 by HTLs, thus impeding the activation of both BLs and CTLs. In addition, UDCA represses the overexpression of MHC-I and, perhaps, MHC-II, as well as ICAM-1 and LFA-1. See the text for more details.

immunoregulatory effects of UDCA may also help to support its alleged prophylactic use in other immune-mediated hepatopathies involving humoral and cellular immune responses, such as acute graft-versus-host disease secondary to haemopoietic stem cell transplantation [200] and liver allograft rejection [201].

Immunosuppressive and anti-inflammatory properties of UDCA

In autoimmune cholestatic liver disorders, such as PBC and PSC, humoral and cellular immune responses are exacerbated. The former is due to the production of antibodies against certain autoantigens against which the patient has lost immunological tolerance. The latter involves the direct attack of hepatocytes and cholangiocytes by CTLs (cytotoxic T-lymphocytes) ($CD8^+$), which have been activated by cytokines produced by HTLs (helper T-lymphocytes) ($CD4^+$), such as IFN-γ (interferon-γ) and IL (interleukin)-1, -2, -4 and -6 [202,203]. These cytokines are involved in the damage of hepatocytes and cholangiocytes by inducing the proliferation and activation of CTLs and natural killer cells [204,205] or, in the case of IFNγ, by causing a direct impairment in cholangiocyte integrity [206].

UDCA is thought to inhibits humoral autoimmunity, as suggested by its ability to suppress the production of IgM, IgG and IgA by B-lymphocytes exposed to bacteria, both from normal individuals and from PBC patients [204]. UDCA also attenuates the cellular immune response by inhibiting the release of cytokines produced by blood mononuclear cells, such as IL-2, IL-4 and IFN-γ [204]. However, it should be noted that endogenous bile acids that accumulate in cholestasis, such as CDCA, have immunosuppressive properties greater than those of UDCA [203,207] and therefore their replacement by UDCA may lead to an even lower immunosuppression. Furthermore, the physiopathological relevance of these *in vitro* studies has been questioned due to shortcomings in mimicking the situation *in vivo*, such as a lack of physiological amounts of proteins in the extracellular medium; this renders UDCA concentrations in the assay higher than those reached in patients and probably toxic for immunocompetent cells [208]. With more realistic conditions (UDCA concentrations in the micromolar

range and in the presence of fetal calf serum), uptake of UDCA by monocytes and Kupffer cells was very low, and had no effect on lipopolysaccharide-induced cytokine release [208]. It remains unclear, however, what level of bile acids is actually reached at the close proximity of the bile ductules where inflammatory cells accumulate in cholestatic cholangiopathies; concentrations of UDCA in the peribiliary plexus circulation higher than systemic ones are expected, due to its efficient cholehepatic shunting (see above). If so, this may explain the decrease in circulating TNF-α and TGF-β1, two cytokines that reflect severity of the disease, in PBC patients taking UDCA [209]. Furthermore, UDCA attenuated concanavalin A-induced mouse inflammatory liver injury by decreasing the release of TNF-α from natural killer T-cells [210]; a similar effect was afforded by glycoUDCA in astrocytes exposed to unconjugated bilirubin [211], suggesting that this may be an ubiquitous mechanism. In contrast, UDCA failed to reduce serum TNF-α levels in PSC patients [212], a factor that might contribute to the lack of a beneficial effect of this drug in PSC progression [13]. UDCA also interferes with TNF-α-mediated activation of NF-κB, at least in part via binding to the glucocorticoid receptor [93]. By doing so, UDCA would break the vicious circle by which TNF-α activates NF-κB, which in turn induces the expression of TNF-α [213]. Again, this beneficial mechanism seems to be ubiquitous. UDCA inhibited NF-κB activation induced by deoxycholic acid in human colon cancer cells [214] or by amyloid β peptide in a microglial cell line [215]. These findings support the suggested applications of UDCA in chemoprotection of colon cancer [216] and Alzheimer's disease [84] respectively.

UDCA-induced reversal of aberrant expression of MHCs

MHCs enable the recognition of autoantigens by lymphocytes involved in the humoral and cellular response in autoimmune diseases. For example, when exposed together with the auto-antigenic epitope, MHC-I activates CTLs ($CD8^+$), whereas MHC-II activates HTLs ($CD4^+$). The latter produces cytokines such as TNF-α and IFN-γ, which increase the expression of MHC-I in non-immunological cells (for example hepatocytes); this increases the possibility of being immunologically attacked. Endogenous bile acids accumulated in cholestatic hepatopathies exacerbate the induction of both MHC-I in hepatocytes and MHC-II in cholangiocytes [217]. UDCA treatment inhibits MHC-I overexpression in PBC patients [218,219]. On the other hand, the ability of UDCA to counteract the overexpression of MHC-II in cholangiocytes is doubtful, since there is evidence either in favour [219] or against [218] this protective mechanism.

The mechanism(s) by which UDCA treatment inhibits MHC overexpression in cholestasis remains to be ascertained. It is possible that this is via the UDCA-induced activation of the glucocorticoid receptor [220], a well-recognized mediator of the suppressive effects of glucocorticoids on the transcription of MHCs [221]. In line with this, UDCA suppressed the expression of MHC-II induced by IFN-γ in ovarian cells stably transfected with the glucocorticoid receptor in a ligand-independent manner [222], although this remains to be confirmed in hepatocytes or cholangiocytes for MHC-I and MHC-II respectively. In addition, UDCA inhibited the CDCA-induced production of IFN-γ in peripheral blood mononuclear cells from PBC patients [223].

The immune-mediated progressive destruction of interlobular bile ducts in PBC requires the penetration of lymphocytes and other inflammatory cells to the peribiliary vascular plexus and portal venules, followed by migration into the perivenular tissue towards the bile ducts. In this process, the increased expression of both ICAM-1 (intercellular adhesion molecule-1) and LFA-1 (lymphocyte function-associated antigen-1) in cholangiocytes and lymphocytes respectively is essential. In PBC, ICAM-1 was not only expressed on the basal, but also on the luminal, side of the plasma membrane of bile duct epithelial cells, whereas LFA-1 was detected in lymphocytes around and among damaged bile duct epithelial cells. UDCA therapy reduces ICAM-1 and LFA-1 expression at all of these sites [224], and this effect was additive with that afforded by corticoid therapy [225].

The immunomodulatory effects of UDCA in PBC were not consistently observed in other autoimmune hepatopathies. For example, UDCA therapy failed to counteract the increase in serum TNF-α and IL-8 levels in PSC patients and the overexpression of MHC-I/MHC-II and ICAM-1 [212]. Therefore the immunomodulatory properties of UDCA should be analysed in the context of each autoimmune hepatic disease in particular.

FUTURE DIRECTIONS

There have been extraordinary advances in the understanding of the anti-cholestatic mechanisms of UDCA over the last few years. This novel knowledge has allowed us to envisage and successfully test new therapeutic applications, even beyond liver disease.

There are certainly many additional beneficial properties of UDCA to be discovered, as well as many details to be revealed on some of its well-established, but poorly understood, therapeutic properties. The present scientific efforts are focused on taking advantage of the more precise knowledge of the molecular events that occur in every disease to design more 'personalized' therapeutic strategies for each situation. In this regard, post-treatment monitoring of the decrease in selective bile acids in serum identified to mark disease severity or the increase in others known to aggravate the disease, including the excessive increase in unconjugated UDCA

to potentials dangerous levels, may help to establish the ideal UDCA dosage for each hepatopathy.

A number of alternative drugs are currently being tested in pre-clinical studies for the treatment of cholestatic disease, including selective modulators of nuclear receptors and signalling pathways thought to mediate cholestasis. It will be difficult, however, to find substitutes more innocuous than UDCA and that combine in just one molecule so many simultaneous salutary effects with similar efficacy. This is particularly critical in cholestatic diseases, since they affect a multifunctional organ like the liver, in which the primary imbalance caused by the retention of potentially toxic metabolites triggers multiple pathophysiological changes that affect the general homoeostasis of the body. On this basis, we predict a still long reign of UDCA as 'the' therapeutic agent of choice in cholestasis.

FUNDING

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