Side Chain Structure Determines Unique Physiologic and Therapeutic Properties of *nor*Ursodeoxycholic Acid in *Mdr2*$^{-/-}$ Mice

Emina Halilbasic,[1] Romina Fiorotto,[2] Peter Fickert,[1] Hanns-Ulrich Marschall,[3] Tarek Moustafa,[1] Carlo Spirli,[2] Andrea Fuchsbichler,[4] Judith Gumhold,[1] Dagmar Silbert,[1] Kurt Zatloukal,[4] Cord Langner,[4] Uday Maitra,[5] Helmut Denk,[4] Alan F. Hofmann,[6] Mario Strazzabosco,[2,7,8] and Michael Trauner[1]

24-*nor*ursodeoxycholic acid (*nor*UDCA), a side chain–modified ursodeoxycholic acid derivative, has dramatic therapeutic effects in experimental cholestasis and may be a promising agent for the treatment of cholestatic liver diseases. We aimed to better understand the physiologic and therapeutic properties of *nor*UDCA and to test if they are related to its side chain length and/or relative resistance to amidation. For this purpose, *Mdr2*$^{-/-}$ mice, a model for sclerosing cholangitis, received either a standard diet or a *nor*UDCA-, tauro*nor*ursodeoxycholic acid (tauro-*nor*UDCA)-, or di*nor*ursodeoxycholic acid (di*nor*UDCA)-enriched diet. Bile composition, serum biochemistry, liver histology, fibrosis, and expression of key detoxification and transport systems were investigated. Direct choleretic effects were addressed in isolated bile duct units. The role of Cftr for *nor*UDCA-induced choleresis was explored in *Cftr*$^{-/-}$ mice. *nor*UDCA had pharmacologic features that were not shared by its derivatives, including the increase in hepatic and serum bile acid levels and a strong stimulation of biliary HCO_3^--output. *nor*UDCA directly stimulated fluid secretion in isolated bile duct units in a HCO_3^--dependent fashion to a higher extent than the other bile acids. Notably, the *nor*UDCA significantly stimulated HCO_3^--output also in *Cftr*$^{-/-}$ mice. In *Mdr2*$^{-/-}$ mice, cholangitis and fibrosis strongly improved with *nor*UDCA, remained unchanged with tauro-*nor*UDCA, and worsened with di*nor*UDCA. Expression of Mrp4, Cyp2b10, and Sult2a1 was increased by *nor*UDCA and di*nor*UDCA, but was unaffected by tauro-*nor*UDCA. *Conclusion:* The relative resistance of *nor*UDCA to amidation may explain its unique physiologic and pharmacologic properties. These include the ability to undergo cholehepatic shunting and to directly stimulate cholangiocyte secretion, both resulting in a HCO_3^--rich hypercholeresis that protects the liver from cholestatic injury. (HEPATOLOGY 2009;49:1972-1981.)

See Editorial on Page 1795

Due to the limited efficacy of current medical treatment of chronic diseases of the biliary tree, such as sclerosing cholangitis,[1] there is an urgent need to develop novel treatment strategies and to test them in animal models for human cholangiopathies.[2] Mice lacking the canalicular phosphatidylcholine floppase *Mdr*2 (*Abcb4*) (*Mdr2*$^{-/-}$ mice) develop chronic cholestatic liver disease including sclerosing cholangitis and biliary fibrosis.[3,4] These unique characteristics of *Mdr2*$^{-/-}$ mice are of particular interest because increasing evidence suggests a key role of *MDR3* (*ABCB4,* the human orthologue of rodent *Mdr2*) defects in human cholestatic liver diseases ranging from neonatal cholestasis to biliary cirrhosis in adults.[5] More recently, *MDR3* variants have been observed in patients with primary sclerosing cholangitis[6] and idiopathic biliary fibrosis,[7] highlighting the importance of *MDR3* as a modifier gene in disease course and severity of cholangiopathies.

Abbreviations: dinorUDCA, dinorursodeoxycholic acid; IBDU, isolated bile duct unit; K19, keratin 19; norUDCA, 24-norursodeoxycholic acid; α-SMA, α-smooth muscle actin; tauro-norUDCA, tauronorursodeoxycholic acid; UDCA ursodeoxycholic acid.

From the [1]Laboratory of Experimental and Molecular Hepatology, Department of Gastroenterology and Hepatology, University Clinic of Internal Medicine, Medical University of Graz, Graz, Austria; the [2]Section of Digestive Diseases, Department of Internal Medicine, School of Medicine, Yale University, New Haven, CT; [3]Karolinska University Hospital Huddinge, Stockholm, Sweden; [4]Institute of Pathology, Medical University of Graz, Graz, Austria; the [5]Department of Organic Chemistry, Indian Institute of Science, Bangalore, India; the [6]Department of Medicine, University of California, San Diego, CA; [7]CeLiveR, Ospedali Riuniti di Bergamo, Bergamo, Italy; and the [8]Department of Clinical Medicine and Prevention, University of Milano-Bicocca, Milan, Italy.

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We have recently demonstrated that cholangitis and biliary fibrosis in *Mdr2*$^{-/-}$ mice are reversed by 24-*nor*ursodeoxycholic acid (*nor*UDCA), a C_{23} homologue of ursodeoxycholic acid (UDCA) with one less methyl group in its side chain,[8] suggesting that side chain–modified bile acids may represent a novel and promising treatment strategy for cholestatic liver diseases. The potential therapeutic mechanisms, hypothesized so far to explain the pharmacodynamic effects of *nor*UDCA in *Mdr2*$^{-/-}$ mice, include increased hydrophilicity (and decreased cytotoxicity) of the circulating bile acid pool, induction of detoxification pathways and elimination routes for bile acids, as well as potential direct anti-inflammatory and anti-fibrotic effects.[8] The therapeutic effects of *nor*UDCA may be related to its side chain structure that strongly influences *nor*UDCA metabolism.[8,9] The metabolic fate of *nor*UDCA differs substantially from that of other natural bile acids, including UDCA, because of its minimal *N*-acyl amidation (conjugation) with taurine or glycine[8,9] together with its considerable hydroxylation and glucuronidation. In contrast to conjugated bile acids, unconjugated *nor*UDCA is a weak organic acid with only two hydroxyl groups that can be reabsorbed from bile by cholangiocytes and subsequently be resecreted by hepatocytes thereby undergoing a cholehepatic shunt, a process that is associated with a HCO_3^--rich hypercholeresis.[10] Flushing of bile ducts by HCO_3^--rich bile may dilute and inactivate toxic biliary contents, thereby protecting bile ducts from further injury in *Mdr2*$^{-/-}$ mice.[8] HCO_3^--rich hypercholeresis has also been described for UDCA (the parent compound of *nor*UDCA), although this process only occurs *in vitro* once taurine stores have become depleted and the concentration of unconjugated UDCA prevails over that of its taurine conjugate.[11] Recent studies have shown that UDCA is also able to directly stimulate fluid secretion by cholangiocytes through Cftr-mediated ATP secretion and apical purinergic signaling,[12] but the potential effects of *nor*UDCA remain to be elucidated.

To test whether the physiologic and therapeutic properties of *nor*UDCA are related to its relative resistance to *N*-acyl amidation or its side chain length, we compared *nor*UDCA with tauro-*nor*UDCA (a synthetic taurine conjugate of *nor*UDCA) and di*nor*UDCA (a C_{22} homologue of UDCA and *nor*UDCA with only three carbon atoms in its side chain) in the *Mdr2*$^{-/-}$ mouse model of sclerosing cholangitis and biliary fibrosis. Moreover, this study was designed to test the hypothesis that the pharmacologic effects of *nor*UDCA depend on its ability to recirculate between bile ducts and hepatocytes as a consequence of cholehepatic shunting and/or its direct stimulatory effects on cholangiocyte secretion. This information should be critical for the design of novel drugs for the treatment of cholestatic liver diseases.

Materials and Methods

Animal Experiments. *Mdr2*$^{-/-}$ mice (FVB/N background) were obtained from Jackson Laboratory (Bar Harbor, ME). Eight-week-old male *Mdr2*$^{-/-}$ mice received either standard chow (Ssniff, Soest, Germany) or a diet containing *nor*UDCA, tauro-*nor*UDCA, or di*nor*UDCA (0.5% wt/wt, the dosage that corresponds approximately to 15 mg/mouse/day) over 4 weeks. Congenic B6.129P2-Cftr^{tm1Unc} mice, which possess the S489X mutation that blocks transcription of Cftr,[13] were fed with Peptamen (Nestle Clinical Nutrition, Deerfield, IL) or with Peptamen containing *nor*UDCA (in a dosage equivalent to that used in *Mdr2*$^{-/-}$ mice) for 1 week. All animals were housed under a 12:12-hour light/dark cycle and permitted *ad libitum* consumption of water and diet. The experimental protocols were approved by the local Animal Care and Use Committees according to criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the U.S. National Academy of Sciences (National Institutes of Health publication 86-23, revised 1985).

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The Medical University of Graz has filed the patent for the use of norUDCA in the treatment of liver diseases, and Michael Trauner, Alan Hofmann, and Peter Fickert are listed as inventors (publication number WO2006119803).

Address reprint requests to: Michael Trauner, M.D., Laboratory of Experimental and Molecular Hepatology, Department of Gastroenterology and Hepatology, Medical University Graz, Auenbruggerplatz 16, 8036 Graz, Austria. E-mail: michael.trauner@meduni-graz.at.

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Synthesis of* norUDCA *(3α,7β-Dihydroxy-5β-cholan-23-oic Acid), tauro-*norUDCA *[3α,7β-Dihydroxy-5β-cholan-23-oic Acid* N*-(2-Sulfoethyl)amide,* norUDC-tau*], and di*norUDCA *(3α,7β-Dihydroxy-5β-cholan-22-oic Acid, bis*norUDCA*). The compounds studied (Supporting Fig. 1) were synthesized from UDCA as described.[14–16] Unconjugated bile acids were pure by thin layer chromatography; tauro-*nor*UDCA was pure by high-pressure liquid chromatography.

Analysis of Bile Flow and Composition. Bile flow and composition were determined as described.[8] Before harvesting, mice were anesthetized with tribromoethanol (Avertin) 250 mg/kg body weight intraperitoneally. The common bile duct was ligated, and the gallbladder was cannulated for collection of bile. After a 10-minute equilibration period, bile was collected for 1 hour in test tubes under mineral oil for determination of HCO_3^-, total carbon dioxide and pH using an automatic blood gas analyzer (AVL 995 hp, AVL, Graz, Austria; COBAS Mira plus, Roche). For bile flow measurements and further bile analysis, bile was collected for the following 30 minutes in preweighed test tubes. Bile flow was determined gravimetrically and normalized to liver weight as described.[17] Biliary glutathione concentration was determined after protein precipitation using 5% metaphosphoric acid (Glutathione Assay Kit; Calbiochem, La Jolla, CA) according to the manufacturer's instructions. Biliary bile acid concentration (of 3α-hydroxy bile acids) was measured enzymatically using a colorimetric bile acid kit (Ecoline S+, Holzheim, Germany).

Intrahepatic Mouse Bile Duct Units and Assessment of Ductular Secretion via Video-Optical Planimetry. Isolated bile duct units (IBDUs)—sealed fragments of isolated bile ductules with retained polarity that can secrete into a closed lumen—were prepared, purified, and cultured as described.[18–20] Secretion in IBDUs is determined by measuring expansion of their lumen over time using video-optical planimetry as an indicator of the ductular secretion rate, as described.[18–20] After a 5-minute baseline period, IBDUs were exposed to bile acids (at 250 μM concentration) for up to 30 minutes, and the luminal area was measured every 5 minutes via video-optical planimetry. Serial images of the IBDUs were acquired as described.[12] To determine whether secretion depends on HCO_3^-, IBDUs were perfused with either HCO_3^--free 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer or HCO_3^--containing Krebs-Ringer buffer.

Bile Acid Analysis. Bile acids were extracted from liver homogenate, serum, and bile and analyzed using electrospray ionization mass spectrometry and gas chromatography mass spectrometry as described.[8,21]

Routine Serum Biochemistry. Alanine aminotransferase and alkaline phosphatase were analyzed with a Hitachi 917 analyzer (Boehringer Mannheim, Mannheim, Germany).

Liver Histology. For conventional light microscopy, formalin-fixed livers were embedded in paraffin, and 4-μm thick sections were stained with hematoxylin-eosin and Sirius red staining. The sections were coded and examined by a pathologist (C. L.) who was unaware of the animals' genotype and treatment.

Immunohistochemistry for Keratin 19. As described, keratin 19 (K19) immunohistochemistry was performed on cryosections with monoclonal rat anti–Troma-III antibody (dilution 1:100, Developmental Studies Hybridoma Bank) developed under the auspices of the National Institute of Child Health and Human Development (NICHID) and maintained at the University of Iowa (Iowa City, IA).[22]

Western Blotting. α-Smooth muscle actin (α-SMA) and K19 protein levels were determined in liver homogenates (30 μg protein) via western blotting as described[8,23] using monoclonal mouse antibody against α-SMA (dilution, 1:1,500; Sigma-Aldrich, Vienna, Austria) and monoclonal rat K19 (dilution, 1:1,500; Developmental Studies Hybridoma Bank). Blots were reprobed with an anti–β-actin antibody (dilution, 1:5,000; Sigma-Aldrich, Vienna, Austria) to confirm the specificity of the observed changes in transporter protein levels.

Determination of Hepatic Hydroxyproline Content. To quantify liver fibrosis, hepatic hydroxyproline was measured as described.[24]

Messenger RNA Analysis. Total hepatic RNA was isolated and reverse-transcribed into complementary DNA as described.[21] In brief, total RNA was isolated using TRIzol Reagent (Invitrogen, Austria) and reverse-transcribed into complementary DNA by using the GeneAmp Gold RNA PCR Core Kit (Applied Biosystems, Vienna, Austria) according to the manufacturer's instructions. Real-time PCR was performed on a GeneAmp 7900 Sequence Detection System with GeneAmp 7900 SDS software (Applied Biosystems, CA). Polymerase chain reaction was performed in a 20-μL reaction mixture containing TaqMan or SYBR Universal PCR Master Mix (Applied Biosystems, CA). Polymerase chain reaction products were checked via gel electrophoresis for correct size and quality. All data were normalized to 18s ribosomal RNA. The TaqMan oligonucleotides were as follows: Mrp4 forward primer 5′-TTAGATGGGCCTCTGGTTC-3′, reverse primer 5′-GCCCACAATTCCAACCTTT-3′, probe 5′-ACTGCGCTCATCAAGTCCAGGG-3′ (GenBank accession number W54702); Cyp2b10 forward

Table 1. Bile Flow and Composition in *Mdr2*$^{-/-}$ Mice under *nor*-Bile Acid Treatment

Variable	Control (n = 8)	*nor*UDCA (n = 8)	T-*nor*UDCA (n = 8)	di*norUDCA* (*n = 4*)
Bile flow (μL/gLW^{-1}/min^{-1})	2.1 ± 0.2	3.4 ± 0.4*	3.1 ± 0.5*	1.9 ± 0.2†
Bile acid output (nmol/gLW^{-1}/min^{-1})	10.1 ± 2.5	21.7 ± 7.4*	18.5 ± 4.9*	10.8 ± 2.0†
Bicarbonate output (nmol/gLW^{-1}/min^{-1})	56.4 ± 4.0	104.4 ± 22.3*	76.8 ± 12.5*,†	50.8 ± 6.7†
Glutathione output (nmol/gLW^{-1}/min^{-1})	3.9 ± 1.2	6.5 ± 1.0*	4.1 ± 0.7*,†	4.7 ± 1.1†

Values are expressed as the mean ± standard deviation. Control, standard chow-fed animals; *nor*UDCA, *nor*UDCA–fed animals; T-*nor*UDCA, tauro-*nor*UDCA–fed animals; di*nor*UDCA, di*nor*UDCA–fed animals.

*$P < 0.05$ versus control.

†$P < 0.05$ versus *nor*UDCA.

primer 5′-CAATGGGGAACGTTGGAAGA-3′, reverse primer 5′-TGATGCACTGGAAGAGGAAC-3′, probe 5′- TTCGTAGATTCTCTCTGGCCACCATGAGA-3′ (GenBank accession number NM_009998); Sult2a1 forward primer 5′-GGAAGGACCACGACTCATAAC-3′, reverse primer 5′-GATTCTTCACAAGGTTTGTGTTACC-3′, probe 5′-CCCATCCATCTCTTCTCCAAGTCTTTCTTCAG-3′ (GenBank accession number L02335). 18sRNA was determined using SYBR Green Mastermix and forward primer 5′-GTAACCCGTTGAACCCCATT-3′, reverse primer 5′-CCATCCAATCGGTAGTAGCG-3′ (GenBank accession number X00686).

Statistical Analysis. Data are reported as the arithmetic mean ± standard deviation of reported animals in each group. Statistical analysis was performed using SPSS software. The data were analyzed with a nonparametric Mann-Whitney U test. A P value <0.05 was considered significant.

Results

nor*UDCA and Its Side Chain Derivatives Have Differing Effects on Bile Flow and Composition.* To test whether side chain modification of *nor*UDCA influenced bile flow and composition in a cholestatic mouse model, we fed *Mdr2*$^{-/-}$ mice, a model for spontaneous sclerosing cholangitis and biliary fibrosis,[4] with *nor*-bile acids. In contrast to the significant increase of bile flow and biliary output of bile acids, HCO_3^- and glutathione by *nor*UDCA treatment in *Mdr2*$^{-/-}$ mice[8] (Table 1), tauro-*nor*UDCA had no effect on glutathione output, and the effects on HCO_3^- output were much less pronounced (Table 1). di*nor*UDCA altered neither bile flow nor bile acid, HCO_3^-, or glutathione output (Table 1). Similar to *Mdr2*$^{-/-}$ mice, *nor*UDCA also stimulated bile flow and bicarbonate output in corresponding wild-type animals as reported.[8] Of note, stimulation of biliary HCO_3^- output in wild-type animals was significantly higher under *nor*UDCA (241.2 ± 45.3 nmol/g liver weight^{-1}/minute^{-1}) compared with tauro-*nor*UDCA (123.6 ± 47.0 nmol/g liver weight^{-1}/minute^{-1}) and untreated controls (44.3 ± 6.5 nmol/g liver weight^{-1}/min^{-1}). These differences between *nor*UDCA and tauro-*nor*UDCA in their ability to induce a HCO_3^--rich bile flow both in normal and cholestatic conditions could be secondary to the potential of *nor*UDCA to undergo cholehepatic shunting[10] and/or stimulate HCO_3^--rich choleresis at the level of the bile ducts [11].

nor*UDCA Significantly Increases Fluid Secretion in IBDUs in a Partially HCO_3^--Dependent Manner.* To directly address the choleretic potential of side chain–shortened derivatives of UDCA at the cholangiocyte level, we perfused mouse IBDUs *in vitro* with *nor*UDCA, tauro-*nor*UDCA, or di*nor*UDCA and measured luminal expansion rates as a marker of cholangiocellular bile secretion.[12] IBDUs perfused with UDCA were used as an internal control as reported.[12] In HCO_3^--containing media, *nor*UDCA significantly increased cholangiocyte fluid secretion in IBDUs above the levels obtained by perfusion with equimolar amounts of tauro-*nor*UDCA and UDCA, while the stimulation of the secretion by di*nor*UDCA was the lowest (Table 2). In HCO_3^--free conditions, bile acid–induced cholangiocyte secretion was drastically reduced, but not abolished. The stimulation of cholangiocyte secretion in the absence of HCO_3^- was similar for both *nor*UDCA and tauro-*nor*UDCA in contrast to previously mentioned differences in HCO_3^--containing medium (Table 2). These results strongly argue for the concept that *nor*UDCA directly stimulates fluid and HCO_3^- secretion in cholangiocytes in addition to stimulating canalicular bile flow.

nor*UDCA-Induced Stimulation of Biliary HCO_3^- Output* In Vivo *Is Partly Cftr-Independent.* To further understand *nor*UDCA's mechanisms of action and to test whether *nor*UDCA-induced HCO_3^--rich hypercholeresis is Cftr-dependent, we fed wild-type and *Cftr* knockout *(Cftr*$^{-/-}$*)* mice with *nor*UDCA for 1 week. In wild-type littermates, *nor*UDCA significantly increased HCO_3^- output (5.2-fold) and bile flow (3.2-fold) (Table 3). In *Cftr*$^{-/-}$ mice *nor*UDCA also stimulated HCO_3^-

Table 2. Effect of *nor*-Bile Acids on Fluid Secretion in IBDUs using HCO_3^-- Containing and HCO_3^--Free Buffer

Variable	% Increase in Luminal Area after 30 Minutes	n	*P* value
HCO_3 buffer			
Control	12 ± 5	9	
UDCA 250 μM	42 ± 13	9	<0.001*
*nor*UDCA 250 μM	65 ± 21	33	<0.001*
			<0.01‡
T-*nor*UDCA 250 μM	49 ± 18	18	<0.001*
			<0.05†
			NS‡
di*nor*UDCA 250 μM	29 ± 12	18	<0.01*
			<0.001†
			<0.05‡
HCO_3^--free HEPES buffer			
Control	15 ± 5	8	
*nor*UDCA 250 μM	31 ± 11	10	<0.01*
T-*nor*UDCA 250 μM	33 ± 7	8	<0.01*
			NS†
di*nor*UDCA 250 μM	21 ± 12	8	NS*
			NS†

Values are expressed as the mean ± standard deviation.

Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; NS, not significant.

*$P < 0.05$ versus control.

†$P < 0.05$ versus *nor*UDCA 250 μM.

‡$P < 0.05$ versus UDCA 250 μM.

output, while the increase in bile flow failed to reach statistical significance ($P = 0.053$). Of note, the degree of stimulation *of* HCO_3^- output (5.8-fold) and bile flow (3.3-fold) by *nor*UDCA in *Cftr*$^{-/-}$ mice was similar to that achieved in wild-type mice. In line with these *in vivo* findings, *nor*UDCA also stimulated fluid secretion in IBDUs from *Cftr*$^{-/-}$ mice, although to a lower extent compared with IBDUs from wild-type animals (data not shown). Collectively, these finding suggest that *nor*UDCA is able to generate a biliary HCO_3^- secretion also in conditions characterized by defective Cftr. Of interest, the stimulation of glutathione output by *nor*UDCA in wild-type animals was abrogated in *Cftr*$^{-/-}$ mice (Table 3). Furthermore, the amount of bile acid output was lower in *Cftr*$^{-/-}$ mice, a finding consistent with the production of less alkaline bile in Cftr-defective animals.

nor*UDCA and tauro*-nor*UDCA Feeding Increases Biliary Bile Acid Hydrophilicity. To investigate whether the replacement of hydrophobic (cytotoxic) bile acids in phospholipid-depleted bile of Mdr2$^{-/-}$ mice by more hydrophilic ones may contribute to the therapeutic mechanisms of *nor*UDCA, we next determined the bile acid composition in *Mdr2*$^{-/-}$ mice treated with *nor*UDCA and its analogues (Table 4). Bile acid feeding did not increase biliary bile acid concentrations compared with controls. Bile acids were efficiently conjugated with taurine in untreated *Mdr2*$^{-/-}$ mice, as no unconjugated bile acids were present. In contrast, unconjugated bile acids were present in all three bile acid–fed groups. In mice receiving *nor*UDCA, about the half of bile acids were present in unconjugated form. In animals receiving tauro-*nor*UDCA, 14% were not taurine–conjugated, and in animals receiving di*nor*UDCA only 7% of biliary bile acids were unconjugated. The presence of taurine-conjugated bile acids in mice receiving *nor*UDCA can be explained by the ability of the ileal transport system to absorb and thereby conserve the small fraction of *nor*UDCA that undergoes taurine conjugation. Conversely, the presence of unconjugated bile acids in mice receiving tauro-*nor*UDCA results from tauro-*nor*UDCA undergoing deconjugation by intestinal bacteria with absorption of *nor*UDCA. The failure of di*nor*UDCA to alter biliary bile acid composition is remarkable. It suggests that di*nor*UDCA is poorly absorbed by the gastrointestinal tract. The only major biotransformation of any of the bile acid was 6β-hydroxylation to form β-muricholic or *nor*-β-muricholic acid (*nor*-β-MCA). Electrospray ionization mass spectrometry could also confirm the excretion of minor amounts of nonamidated *nor*UDCA- and *nor*-β-MCA-glucuronides and sulfates.

nor*UDCA Is Highly Enriched in Liver and Serum of Mdr2*$^{-/-}$ *Mice.* As shown in Table 4, concentrations of bile acids in serum and liver were 4 to 5 times higher in the *nor*UDCA group compared with controls, whereas

Table 3. Bile Flow and Composition in Wild-Type and *Cftr*$^{-/-}$ Mice with or without *nor*UDCA Treatment

	Wild-Type		*Cftr*$^{-/-}$	
Variable	Control (n = 4)	*nor*UDCA (n = 4)	Control (n = 3)	*nor*UDCA (n = 5)
Bile flow (μL/gLW^{-1}/min^{-1})	0.8 ± 0.2	2.6 ± 0.6*	0.6 ± 0.4	2.0 ± 0.8
Bile acid output (nmol/gLW^{-1}/min^{-1})	17.9 ± 4.7	34.4 ± 9.2	10.9 ± 7.9	18.9 ± 6.0†
Bicarbonate output (nmol/gLW^{-1}/min^{-1})	24.3 ± 6.0	127.2 ± 40.5*	14.3 ± 6.5	83.2 ± 39.3*
Glutathione output (nmol/gLW^{-1}/min^{-1})	3.1 ± 0.7	5.3 ± 1.2*	2.5 ± 2.1	2.7 ± 1.2†

Mice were fed a control diet or a diet containing *nor*UDCA for 1 week. Values are expressed as the mean ± standard deviation.

*$P < 0.05$ wild-type *nor*UDCA versus wild-type control, *Cftr*$^{-/-}$ *nor*UDCA versus *Cftr*$^{-/-}$ control.

†$P < 0.05$ *Cftr*$^{-/-}$ control versus wild-type control, *Cftr*$^{-/-}$ *nor*UDCA versus wild-type *nor*UDCA.

Table 4. Bile Acid Concentration in Serum, Liver, and Bile: Biliary Bile Acid Composition In *Mdr2*$^{-/-}$ Mice under *nor*-Bile Acid Treatment

Variable	Control (n = 6)	*nor*UDCA (n = 6)	T-*nor*UDCA (n = 6)	di*nor*UDCA (n = 4)
Serum (μmol/L)	57 ± 12	313 ± 80*	95 ± 41†	60 ± 10†
Liver (nmol/g)	69 ± 27	307 ± 81*	106 ± 40†	109 ± 45†
Bile (mmol/L)	4.3 ± 1.4	4.2 ± 2.2	3.8 ± 2.3	2.9 ± 0.5
Biliary bile acids (rel. %)				
Taurine-amidated				
Cholic	36.9 ± 0.6	8.1 ± 0.9	12.1 ± 2.1	35.4 ± 0.9
Allocholic	<0.1	<0.1	<0.1	2.3 ± 0.9
Chenodeoxycholic	3.9 ± 0.6	<0.1	<0.1	3.9 ± 0.3
Ursodeoxycholic	0.6 ± 0.3	0.8 ± 0.4	1.1 ± 0.6	0.6 ± 0.3
β-Muricholic	40.2 ± 1.7	4.4 ± 0.7	3.8 ± 0.6	34.0 ± 0.9
α-Muricholic	3.4 ± 0.6	1.3 ± 1.1	0.3 ± 0.2	8.4 ± 0.9
ω-Muricholic	6.2 ± 1.6	0.6 ± 0.4	0.6 ± 0.4	5.7 ± 0.3
*nor*UDCA	<0.1	23.1 ± 1.2	56.2 ± 5.5	<0.1
norβ-Muricholic	<0.1	11.0 ± 1.1	11.7 ± 1.9	<0.1
Glucuronidated, non-amidated				
*nor*diol	<0.1	5.6 ± 2.3	2.4 ± 2.1	<0.1
*nor*triol	<0.1	3.1 ± 1.5	0.8 ± 0.7	<0.1
Non-amidated, non-glucuronidated				
*nor*UDCA	<0.1	13.2 ± 0.9	0.2 ± 1.4	<0.1
norβ-Muricholic	<0.1	17.1 ± 2.4	0.4 ± 0.4	<0.1
*nor*ol-one	<0.1	9.0 ± 2.3	8.4 ± 2.7	<0.1
*nor*diol-sulfate	<0.1	1.5 ± 1.1	0.4 ± 0.4	<0.1
di*nor*UDCA	<0.1	<0.1	<0.1	6.4 ± 0.5
di*nor*ol-one	<0.1	<0.1	<0.1	0.4 ± 0.3

Control, standard chow-fed animals. T-*nor*UDCA, T-*nor*UDCA-fed animals. Values are expressed as the mean ± standard deviation. Values do not add up to 100% because trace bile acids (1%) are not shown.

*$P < 0.05$ versus control.

†$P < 0.05$ versus *nor*UDCA.

bile acid levels did not change after tauro-*nor*UDCA and di*nor*UDCA feeding (Table 4). Similar to bile, 43% to 50% of liver and serum *nor*UDCA and its metabolites were nonamidated. The composition of serum and liver bile acids in the other groups did not differ significantly from that found in the bile (data not shown).

nor*UDCA Is Superior to tauro-*nor*UDCA and di-nor*UDCA in Improving Cholestatic Liver Injury in *Mdr2*$^{-/-}$ Mice. We next determined the impact of these differential pharmacologic mechanisms on the therapeutic efficacy of the *nor*-bile acids on cholestatic liver injury in *Mdr2*$^{-/-}$ mice. *nor*UDCA feeding significantly improved serum alanine aminotransferase and alkaline phosphatase levels (Table 5) and liver histology (Fig. 1 and Supporting Fig. 2) as described.[8] In contrast to *nor*UDCA, the reduction of alanine aminotransferase and alkaline phosphatase levels in response to tauro-*nor*UDCA feeding was less pronounced and di*nor*UDCA treatment worsened liver biochemistry (Table 5). Periductal fibrosis on hematoxlin-eosin and Sirius red staining was markedly reduced by *nor*UDCA treatment (Fig. 1A and Supporting Fig. 2); in agreement, α-SMA protein levels were reduced as well (Fig. 1B). In contrast, tauro-*nor*UDCA and di*nor*UDCA had only negligible effects on α-SMA expression levels (Fig. 1B). Increased hepatic hydroxyproline content (205 ± 61 μg/g liver) in *Mdr2*$^{-/-}$ mice, reflecting liver fibrosis, was significantly reduced by *nor*UDCA treatment (129 ± 25 μg/g liver), whereas tauro-*nor*UDCA and di*nor*UDCA did not have a significant effect on liver hydroxyproline levels (172 ± 47 and 187 ± 96 μg/g liver, respectively). *nor*UDCA feeding reversed the ductular reaction, as evidenced by significantly lower hepatic K19 protein levels, serving as a specific cholangiocyte marker (Fig. 2A). On the other hand, tauro-*nor*UDCA had no effect on the ductular reaction (Fig. 2A), and di*nor*UDCA increased ductular mass, most

Table 5. Effects of *nor*-Bile Acids on Liver Enzymes in *Mdr2*$^{-/-}$ Mice

Variable	Control (n = 10)	*nor*UDCA (n = 8)	T-*nor*UDCA (n = 9)	di*nor*UDCA (n = 7)
ALT (U/L)	395 ± 101	272 ± 35*	331 ± 100	903 ± 177*,†
AP (U/L)	212 ± 33	170 ± 22*	188 ± 34	940 ± 175*,†

Control, standard chow-fed animals; *nor*UDCA, *nor*UDCA–fed animals; T-*nor*UDCA, tauro-*nor*UDCA–fed animals; di*nor*UDCA, di*nor*UDCA–fed animals. Values are expressed as the mean ± standard deviation.

*$P < 0.05$ versus control.

†$P < 0.05$ versus *nor*UDCA.

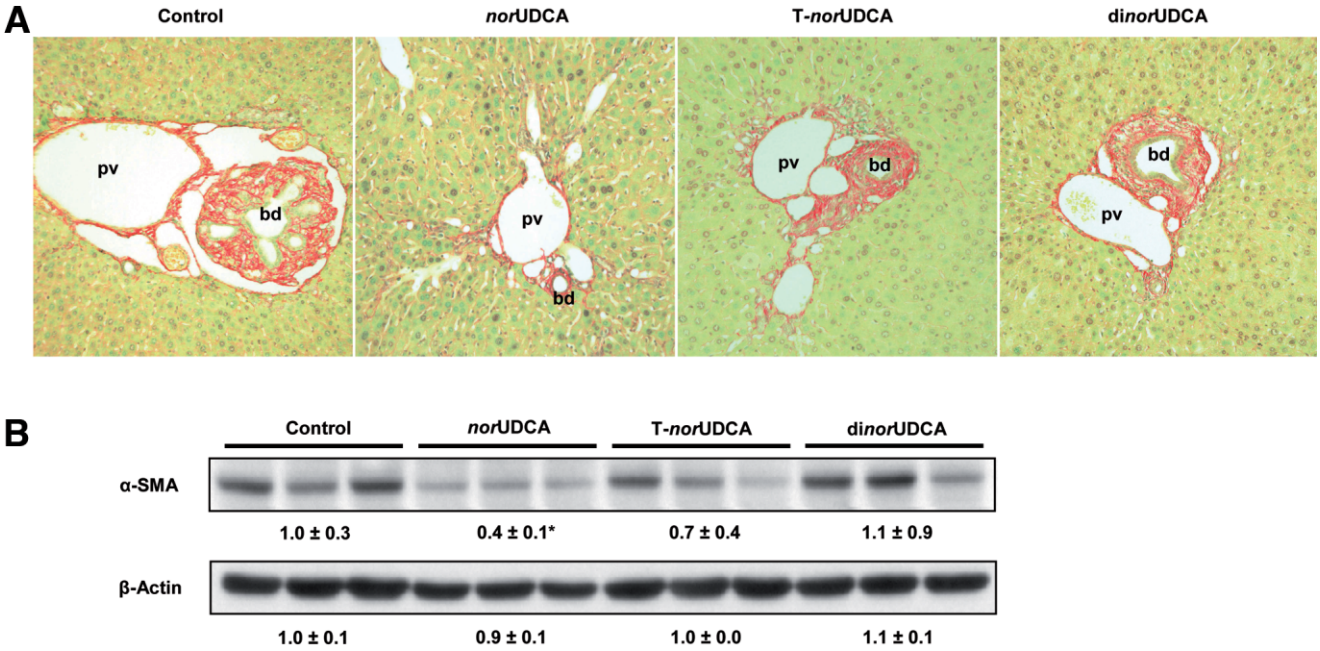

Fig. 1. *nor*UDCA is more effective than tauro-*nor*UDCA and di*nor*UDCA in reducing biliary fibrosis in *Mdr2*$^{-/-}$ mice. (A) Sirius red staining of liver in standard chow-fed (control), *nor*UDCA-fed, tauro-*nor*UDCA-fed (T-*nor*UDCA), and di*nor*UDCA-fed *Mdr2*$^{-/-}$ mice. Compared with striking reduction of fibrosis with periductal collagen fibers (red) in *nor*UDCA-treated *Mdr2*$^{-/-}$ mice, the effects of tauro-*nor*UDCA and di*nor*UDCA were much weaker (magnification ×20). bd, bile duct; pv, portal vein. (B) Western blot analysis of hepatic α-SMA protein levels (marker for activated myofibroblasts) in liver homogenates of standard chow-fed (control), *nor*UDCA-fed, tauro-*nor*UDCA-fed (T-*nor*UDCA), and di*nor*UDCA-fed *Mdr2*$^{-/-}$ mice. Densitometry data are expressed as n-fold change relative to standard chow-fed *Mdr2*$^{-/-}$ mice. Values are expressed as the mean ± standard deviation. Note a significant decrease in α-SMA protein levels by *nor*UDCA treatment. *$P < 0.05$ versus control.

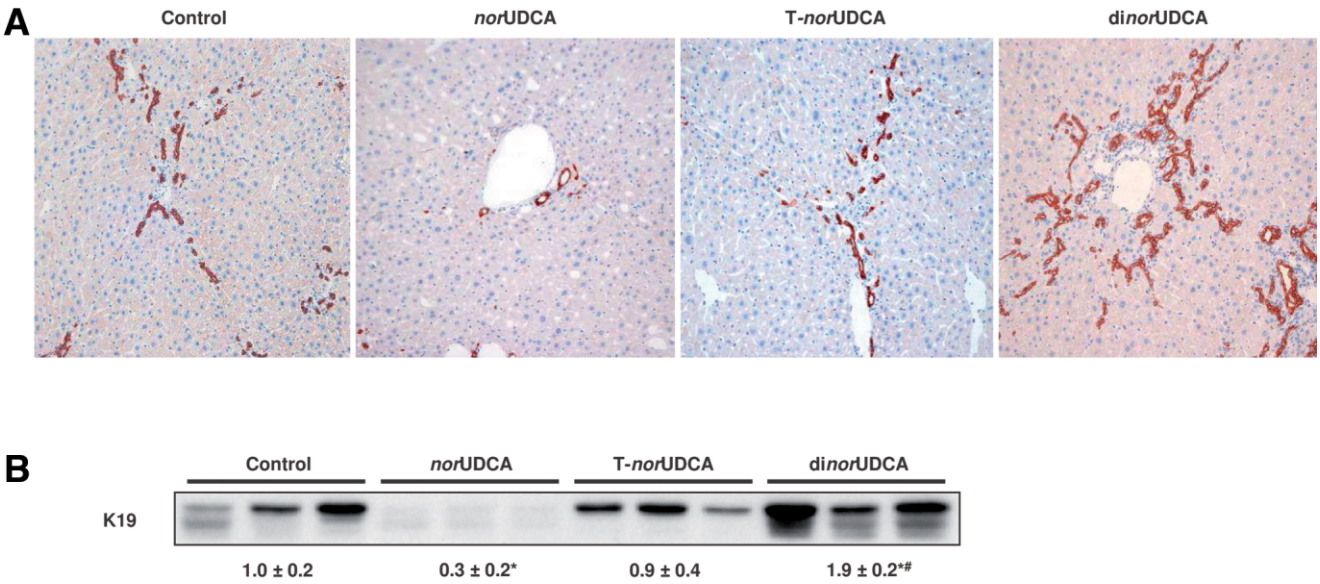

Fig. 2. *nor*UDCA, but not tauro-*nor*UDCA and di*nor*UDCA, reduce ductular proliferation in *Mdr2*$^{-/-}$ mice. (A) Immunohistochemistry of cholangiocytes using an anti-K19 antibody in standard chow-fed (control), *nor*UDCA-fed, tauro-*nor*UDCA-fed (T-*nor*UDCA), and di*nor*UDCA-fed *Mdr2*$^{-/-}$ mice (magnification ×20). Ductular proliferation in standard chow-fed *Mdr2*$^{-/-}$ mice was reduced by *nor*UDCA and remained unchanged after tauro-*nor*UDCA. di*nor*UDCA even increased ductular proliferation in *Mdr2*$^{-/-}$ mice. (B) Western blot analysis of hepatic K19 protein levels in liver homogenates of standard chow-fed (control), *nor*UDCA-fed, tauro-*nor*UDCA-fed (T-*nor*UDCA), and di*nor*UDCA-fed *Mdr2*$^{-/-}$ mice. Densitometry data are expressed as n-fold change relative to standard chow-fed *Mdr2*$^{-/-}$ mice. Values are expressed as the mean ± standard deviation. Note the significant decrease in K19 protein levels by *nor*UDCA and significant increase by di*nor*UDCA. *$P < 0.05$ versus control. $^{\#}P < 0.05$ versus *nor*UDCA.

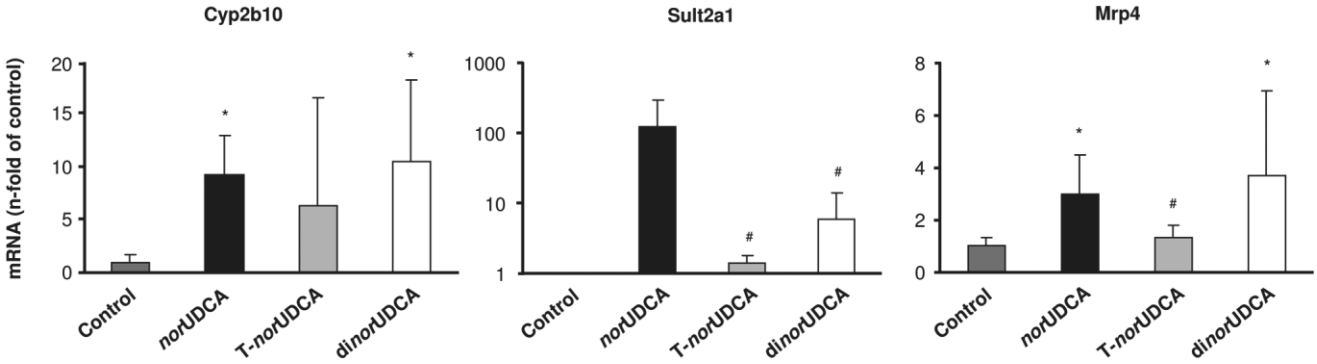

Fig. 3. Effects of *nor*-bile acids on hepatic messenger RNA expression of phase I and II detoxification enzymes and alternative bile acid transporter. Relative messenger RNA expression of Cyp2b10, Sult2a1, and Mrp4 of standard chow-fed (control), *nor*UDCA-fed, tauro-*nor*UDCA-fed (T-*nor*UDCA), and di*nor*UDCA-fed *Mdr2*$^{-/-}$ mice. Values are expressed as n-fold change compared with the control group (mean ± standard deviation of five animals). *nor*UDCA and di*nor*UDCA induced both detoxification enzymes Cyp2b10 and Sult2a1 as well as alternative export pump Mrp4. The effects of T-*nor*UDCA were less pronounced. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus *nor*UDCA.

likely as a result of aggravation of liver injury. Western blotting for K19 (Fig. 2B) demonstrated a significantly reduced expression by *nor*UDCA, no effects by tauro-*nor*UDCA, and an increase in K19 protein mass by di-*nor*UDCA (Fig. 2B).

Effects of* nor*UDCA and Its Side Chain Derivatives on Bile Acid Detoxification and Alternative Export. To study the possible role of induction of bile acid detoxifying enzymes and adaptive transporter expression, we determined messenger RNA expression levels of key phase I (Cyp2b10) and phase II (Sult2a1) detoxifying enzymes as well as phase III export system Mrp4, because these gene products have been identified as key targets of *nor*UDCA.[8] *Cyp2b10*, *Sult2a1*, and *Mrp4* were highly up-regulated in response to *nor*UDCA treatment (Fig. 3), whereas induction by tauro-*nor*UDCA was less pronounced. In contrast to its toxic effects on cholestatic liver injury, di*nor*UDCA feeding resulted in a robust increase of *Cyp2b10*, *Sult2a1*, and *Mrp4* (Fig. 3).

Discussion

*nor*UDCA has been shown to cure liver injury in *Mdr2*$^{-/-}$ mice.[8] Comparing different *nor*UDCA analogues, we now show that the therapeutic properties of *nor*UDCA in the *Mdr2*$^{-/-}$ mouse model for sclerosing cholangitis and portal fibrosis are unique and not shared by its analogues tauro-*nor*UDCA and di*nor*UDCA. Most importantly, we demonstrate that the unique pharmacodynamic effects of *nor*UDCA depend on its relative resistance to taurine conjugation, its ability to undergo cholehepatic shunting with stimulation of canalicular bile flow, and its direct stimulation of cholangiocyte secretion.

The therapeutic effects of *nor*UDCA are highly specific. Conjugation with taurine (resulting in tauro-*nor*UDCA), as well as further side chain shortening (i.e., di*nor*UDCA) resulted in a marked reduction or even complete abrogation of its therapeutic properties, respectively. Our data indicate that the effectiveness of *nor*UDCA in comparison with the other UDCA derivatives correlates with its ability to induce a hydrophilic, HCO_3^--rich choleresis of both canalicular and ductal origin that may flush the injured bile ducts.[8] Previous findings by Yoon et. al. on the choleretic properties of *nor*UDCA in three rodent species[10] indicate that *nor*UDCA may undergo cholehepatic shunting. This multistep process includes reabsorption of the unconjugated protonated, lipophilic bile acid by cholangiocytes, and transport into the periductal capillary plexus, which drains into sinusoids leading to hepatocellular reuptake and canalicular re-excretion into bile. A key physicochemical property of bile acids that undergo cholehepatic shunting is that they are weak organic acids that are easily protonated and are able to cross cell membranes rapidly, presumably by a passive mechanism. Taurine conjugation results in a bile acid being present as a charged anion that can only undergo cholehepatic shunting via apical sodium-dependent bile acid transporter Asbt,[25] as taurine conjugation renders bile acids membrane-impermeable, thus limiting the HCO_3^- generation in bile. Cholehepatic shunting of bile acids may therefore ensue when taurine stores are depleted or conjugation-resistant bile acids are administered.[10] In our experimental conditions, the concept of cholehepatic shunting is further supported by the abundance of unconjugated *nor*UDCA in the liver. These data therefore underline the concept that the therapeutic superiority of *nor*UDCA may indeed be related to its relative resistance to conjugation, as well as its capacity to undergo cholehepatic shunting and to stimulate canalicular bile flow and HCO_3^--rich hypercholeresis.

As shown previously, UDCA, the parent compound of *nor*UDCA, directly stimulates fluid secretion in isolated cholangiocytes through a Cftr-dependent secretion of ATP that, in turn, interacts with apical purinergic receptors, which then stimulate Ca^{2+}-dependent Cl^- channels to secrete Cl^-. The secreted Cl^- is subsequently exchanged with HCO_3^- by the apical anion exchanger AE2.[12] To study whether *nor*UDCA has direct choleretic effects on cholangiocytes, we measured the secretory effects of the three bile acids by quantifying luminal expansion in IBDUs. In line with the choleretic effect observed *in vivo*, *nor*UDCA had the strongest effect on ductular secretion in IBDUs. Secretory effects of *nor*-bile acids were for the most part HCO_3^--dependent. Incubation of IBDUs with a HCO_3^--free buffer reduced *nor*UDCA-stimulated fluid secretion by more than half, by one third for tauro-*nor*UDCA and minimally for di*nor*UDCA. Investigating the mechanisms of these additional HCO_3^--independent mechanisms was, however, beyond the scope of the present study.

Further experiments with *nor*UDCA in *Cftr*$^{-/-}$ mice now indicate that *nor*UDCA-induced HCO_3^- secretion is partly Cftr-independent. This stimulation of biliary HCO_3^- excretion by *nor*UDCA via an at least partly Cftr-independent mechanism should encourage the use of *nor*UDCA as a potential therapeutic strategy in cystic fibrosis-associated hepatobiliary disease. Reduced bile acid output in *nor*UDCA-treated *Cftr*$^{-/-}$ mice is likely due to the lower biliary pH that promotes protonation and reabsorption of *nor*UDCA. This observation also provides an explanation as to why hypercholeretic bile acids directly stimulate HCO_3^-secretion in cholangiocytes. It is attractive to speculate that the mechanisms could have evolved to improve the biliary disposal of potentially toxic weak organic acid compounds that otherwise would continue to recirculate in the liver. In fact, HCO_3^- secretion by bile ducts would be predicted to reduce their protonation and increase the biliary disposal of cholephiles.

The unique combination of cholehepatic shunting (which induces canalicular bile flow) and direct induction of a HCO_3^--dependent secretion from cholangiocytes[8] may exert a flushing effect on the injured biliary tree, likely removing and inactivating toxic compounds. In contrast to the beneficial choleretic effects of *nor*UDCA, UDCA-induced choleresis has been shown to result in disruption of cholangioles and aggravation of liver injury in the *Mdr2*$^{-/-}$ model for sclerosing cholangitis.[4] The underlying mechanism for these puzzling differences remains to be resolved, but could include differences in the capability of directly stimulating ductular secretion. Nevertheless, future clinical trials should take into account potential detrimental *nor*UDCA effects in the context of frank biliary obstruction.

The effects of different *nor*-bile acids on liver fibrosis were closely linked to their effects on ductular reaction, which has been postulated to trigger biliary fibrosis in mice and humans.[22,26,27] Also in our studies the amount of liver fibrosis in treated animals correlated with the amount of ductular reaction. *nor*UDCA reduced both ductular proliferation and biliary fibrosis. Although *nor*UDCA reversed only the pathologically enhanced ductular hyperproliferation in *Mdr2*$^{-/-}$ mice and never induced ductopenia in wild-type animals, we cannot definitely rule out the theoretical risk for development of ductopenia in humans with vanishing bile duct syndromes. Future clinical pilot trials should therefore consider only patients without advanced ductopenia. In comparison to *nor*UDCA, di*nor*UDCA-treated *Mdr2*$^{-/-}$ mice showed a brisk induction of the ductular reaction that likely reflects a regenerative response to increased liver injury, as suggested by significantly increased serum aminotransferases. In contrast to the findings in *Mdr2*$^{-/-}$ mice, wild-type mice fed with the same dosage of di*nor*UDCA with similar hepatic enrichment had induced bile flow, but did not show any alterations in liver enzymes or liver histology excluding a general toxic effect (data not shown).

Bile acids are able to directly stimulate their metabolism and export by the induction of hepatic phase I and phase II detoxification systems as well as by induction of basolateral and apical bile acid transporters.[28] These mechanisms, promoting detoxification of bile acids to more water-soluble derivatives and facilitating their excretion via the kidney, could counteract the effects of cholestasis and liver injury.[28] In line with a previous study,[8] *nor*UDCA feeding resulted in a robust induction of phase I and phase II detoxification enzymes, explaining the increased appearance of polyhydroxylated, sulfated, and glucuronidated *nor*UDCA metabolites in bile, serum, and urine in mice and in humans.[8,9] Similar biotransformation patterns seem to be common to other *nor*-bile acids as shown previously for *nor*chenodeoxycholic and di*nor*chenodeoxycholic acid, both being amidation-resistant and glucuronidated in the hamster and the rat.[29] Taurine conjugation abrogated the effects of *nor*UDCA on induction of *Cyp2b10*, *Sult2a1*, and the basolateral export pump *Mrp4*. Astonishingly, di*nor*UDCA effects on expression of detoxifying and export systems were comparable with those of *nor*UDCA, despite its toxic effect in *Mdr2*$^{-/-}$ mice. Therefore, the observed induction of detoxifying and alternative export systems is not likely to represent a central therapeutic mechanism, and could rather support

a secondary response to injury or to the accumulation of *nor*-bile acids.

In conclusion, this comprehensive analysis of the differential physiologic and therapeutic mechanisms of *nor*UDCA and its derivatives tauro-*nor*UDCA and di-*nor*UDCA clearly demonstrates the therapeutic superiority of *nor*UDCA in an animal model for sclerosing cholangitis and biliary fibrosis (Supporting Table 1). The differential effects of the various *nor*-bile acids depend on specific properties that affect their ability to undergo cholehepatic shunting, thereby stimulating canalicular bile flow as well as by directly stimulating fluid secretion by cholangiocytes. These data may have high relevance for the design of novel pharmacologic treatments for cholangiopathies and liver diseases in general.

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