Research Article

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Differential effects of *nor*UDCA and UDCA in obstructive cholestasis in mice

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Background & Aims: The quest for effective drugs to treat cholangiopathies led to the development of *nor*UDCA previously shown to have potent choleretic effects and to heal cholangiopathy in *Abcb4* knockout (*Abcb4*$^{-/-}$) mice. Its mother compound UDCA had detrimental effects in common bile duct ligated (CBDL) mice, presumably related to its choleretic effects. *nor*UDCA choleretic effects may therefore raise safety concerns when used in cholangiopathies with biliary obstruction. We therefore aimed at comparing the effects of UDCA and *nor*UDCA in clear-cut obstructive cholestasis.

Methods: 0.5% UDCA- or *nor*UDCA-fed wild type and *Abcb4*$^{-/-}$ mice were subjected to CBDL or selective bile duct ligation (SBDL) and compared to controls with regard to liver injury. Bile flow, bile composition, and biliary manometry were compared in UDCA-fed, *nor*UDCA-fed and control mice. Toxicity of UDCA and *nor*UDCA was compared *in vitro*.

Results: Compared to UDCA, liver injury in CBDL mice was significantly lower in almost all *nor*UDCA groups. In SBDL mice, only UDCA induced bile infarcts in the ligated lobes, whereas *nor*UDCA even ameliorated liver injury. *In vitro*, UDCA induced cellular ATP depletion and was significantly more toxic than *nor*UDCA in HepG2 cells, mouse bile duct epithelial cells, and primary human hepatocytes.

Conclusions: Compared to *nor*UDCA, UDCA is significantly more toxic in CBDL mice. *nor*UDCA, in contrast to UDCA, significantly ameliorates liver injury in SBDL mice. Our findings uncover profound differences in metabolism and therapeutic mechanisms of both bile acids with important clinical consequences.

Introduction

Cholangiopathies frequently lead to biliary fibrosis and cirrhosis with the complications of end-stage liver disease [1–6]. This group of liver diseases still represents a major indication for liver transplantation [6–12], underscoring the limited efficacy of currently available medical treatments in cholangiopathies and the urgent need for novel pharmaceutical strategies [4,6,13,14]. UDCA, at present the only approved drug for PBC, appears to exert its beneficial effects by stimulation of bile flow, rendering bile composition less toxic, and reducing the retention of potentially toxic bile acids in hepatocytes and liver injury [5,15–18]. However, the efficacy of UDCA in different cholangiopathies, such as PSC and SSC, is limited [4,6,19]. Alan Hofmann's elegant cholehepatic shunting concept led to the design of side chain-shortened *nor*UDCA with substantial different physicochemical and physiological properties compared to its mother compound UDCA [20–24]. *nor*UDCA was previously also shown to have superior therapeutic effects in *Mdr2/Abcb4* knockout mice (*Abcb4*$^{-/-}$) as model for sclerosing cholangitis [Supplementary Refs. 25–27]. Moreover, *nor*UDCA has potent cholerertic effects in rodents [Supplementary Refs. 26,27] and humans [Supplementary Ref. 28]. Therefore, *nor*UDCA is about to undergo further clinical development for cholangiopathies.

BDL represents the extreme variant of obstructive cholestasis and a well-characterized rodent model system to study the pathophysiology of cholestatic liver disease [Supplementary Refs. 29,30]. We previously demonstrated increased liver injury with

Keywords: Bile acids; Bile infarcts; Biliary pressure; Bile duct epithelial cells; Cholestasis; Cholestatic liver injury; Cholangiopathy; Hepatic transport; Liver injury; Obstructive jaundice; *nor*UDCA; UDCA; Main bile duct stricture; Primary sclerosing cholangitis.

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Abbreviations: Abcb4$^{-/-}$, multidrug resistance gene 2/phospholipid floppase knockout mice; ALT, alanine aminotransferase; AP, alkaline phosphatase; BECs, bile duct epithelial cells; CBDL, common bile duct ligation; CA, cholic acid; SBDL, selective bile duct ligation; *nor*UDCA, 24-nor-ursodeoxycholic acid; UDCA, ursodeoxycholic acid.

aggravation of bile infarcts in UDCA-fed CBDL mice [Supplementary Ref. 31] and postulated that this may primarily be related to increased biliary pressure due to the choleretic effects of UDCA leading to the rupture of the canals of Herring [Supplementary Ref. 31]. Consequently, ductular bile (with millimolar concentrations of bile acids) leaking into the liver parenchyma leads to bile infarcts with oncotic hepatocyte cell death. Comparable findings were obtained in UDCA-fed *Abcb4*$^{-/-}$ mice with partial biliary obstruction as a result of sclerosing cholangitis, but *Abcb4*$^{-/-}$ mice have even increased bile flow, and this model represents, at least from a biliary physiology point of view, no clear-cut situation with complete obstructive cholestasis [Supplementary Ref. 31]. In this model system, UDCA feeding also significantly increased the number and size of bile infarcts in a dose-dependent manner [Supplementary Ref. 31]. Our concept was further supported by amelioration of liver injury in CBDL *FXR* knockout (*FXR*$^{-/-}$) mice, which may be attributed to lower biliary pressure and a more hydrophilic bile acid pool in this genotype [Supplementary Refs. 32–34]. Consequently, CBDL *FXR*$^{-/-}$ mice lacked bile infarcts and showed considerably reduced ductular reaction [Supplementary Ref. 32], both mainly triggered by increased biliary pressure in CBDL rodents [Supplementary Refs. 35,36]. This concept is in contrast with the beneficial effects of *nor*UDCA in *Abcb4*$^{-/-}$ mice despite superior choleretic effects compared to UDCA [Supplementary Refs. 26,27]. These findings may therefore challenge our initial hypothesis that aggravation of bile infarcts in UDCA-fed CBDL and *Abcb4*$^{-/-}$ mice is primarily caused by biliary pressure [Supplementary Refs. 31,37]. Consequently, the aim of the current study was a face-to-face comparison of UDCA and *nor*UDCA therapeutic mechanisms in the extreme variant of obstructive cholestasis with the aid of the CBDL and selective bile duct ligated (SBDL) mouse model. Since *nor*UDCA represents a promising new drug for cholangiopathies with significant obstructive components, information on its effects in clear-cut obstructive cholestasis animal models should be of great value.

Materials and methods

Animal experiments

Experiments were performed in 2-month-old male Swiss albino and C57/BL6 mice (25–30 g), since Swiss albino mice were used in our previous studies and C57/BL6 mice are most frequently used for knockout mouse strain generation. Animals were housed with a 12:12 hour light:dark cycle and permitted *ad libitum* consumption of water. The experimental protocols where approved by the local animal Care and Use Committees (BMWF-66.010/0045-II/10b/2010).

Bile acid feeding

Mice were either fed 0.5% *nor*UDCA- or 0.5% UDCA-supplemented diet, 7 days prior to surgery or following surgery (Fig. 1A), and compared to chow-fed controls since this is the best characterized dose in mice. *nor*UDCA was obtained from Dr. Falk Pharma (Freiburg, Germany), and UDCA from Sigma (Taufkirchen, Germany). CBDL experiments were performed for both mouse strains. SBDL experiments were performed in Swiss albino, *Abcb4*$^{-/-}$ mice and respective WT controls, since in this strain the left hepatic bile duct is easier to isolate and ligate.

Common bile duct ligation (CBDL), selective bile duct ligation (SBDL), mouse harvesting, and serum biochemical analysis were performed as described previously in detail [Supplementary Ref. 31].

Biliary physiology: bile flow measurement, bile composition, and biliary manometry

Separate groups of mice were either fed 0.5% *nor*UDCA-, 0.5% UDCA-supplemented diet or chow diet for 3 days and biliary physiology was determined as described [Supplementary Refs. 32,38].

Statistical analysis

Data are reported as arithmetic means ± SD of 3–8 animals in each group (Fig. 1A). Statistical analysis included Student's *t*-test when appropriate or analysis of variance with Bonferroni *post hoc* testing when more than two groups were compared, using the Sigmastat statistics (Jandel Scientific, San Rafael, CA). $p < 0.05$ was considered significant.

Additional Materials and methods are provided in the Supplementary Data section.

Results

UDCA and norUDCA both significantly increase biliary pressure in mice

To determine the effects of both bile acids on biliary pressure in a situation of complete bile duct obstruction, mice were fed UDCA-supplemented, *nor*UDCA-supplemented, or chow diet for 7 days, a catheter was inserted into the gall bladder, and thereafter the common bile duct was occluded by a suture. After equilibrium of the system (i.e., constant biliary pressure after 8–10 min defined as the basal biliary pressure), it was occluded and biliary pressure was recorded continuously over a 17-min period, until reaching a plateau phase, and compared to chow-fed controls (Fig. 1B). Compared to the UDCA-fed group (black lozenges), biliary pressure in *nor*UDCA-fed mice (gray squares) tended to be higher without reaching statistical significance, but significant differences in comparison to chow-fed mice (open triangles) were reached at an earlier time point in *nor*UDCA-fed mice compared to UDCA-fed mice (Fig. 1B).

norUDCA effects on biliary bicarbonate secretion are superior compared to UDCA

For direct comparison of the effects of UDCA and *nor*UDCA on bile formation, mice were fed 0.5% bile acid-supplemented or chow diet and bile was collected and analyzed. Both bile acids induced bile flow (Table 1), which tended to be even higher in *nor*UDCA-fed mice compared to UDCA-fed animals. UDCA significantly increased biliary bile acid and bicarbonate concentration and consequently the output, whereas increased bile flow in *nor*UDCA-fed mice was primarily related to increased bicarbonate excretion, which was significantly higher compared to UDCA-fed mice (Table 1). It is important to note that biliary bile acid concentration in *nor*UDCA-fed mice was significantly lower than in UDCA-fed mice (approximately the half) and comparable to chow-fed animals (Table 1).

Taken together, these findings clearly show that (i) both bile acids significantly increase biliary pressure to a comparable level in mice, (ii) UDCA significantly induces bile acid-dependent and bile acid-independent bile flow, (iii) *nor*UDCA exclusively stimulates bicarbonate-dependent bile flow.

In contrast to UDCA, norUDCA does not aggravate liver injury in CBDL mice

Based on our current manometric findings, we hypothesized that *nor*UDCA would at least equally aggravate bile infarcts in CBDL mice, due to its potent choleretic properties [Supplementary Refs. 31,37]. To test this hypothesis, CBDL mice were fed a 0.5% bile acid-supplemented diet for 7 days (Group A) and compared to chow-fed CBDL controls. UDCA significantly increased liver

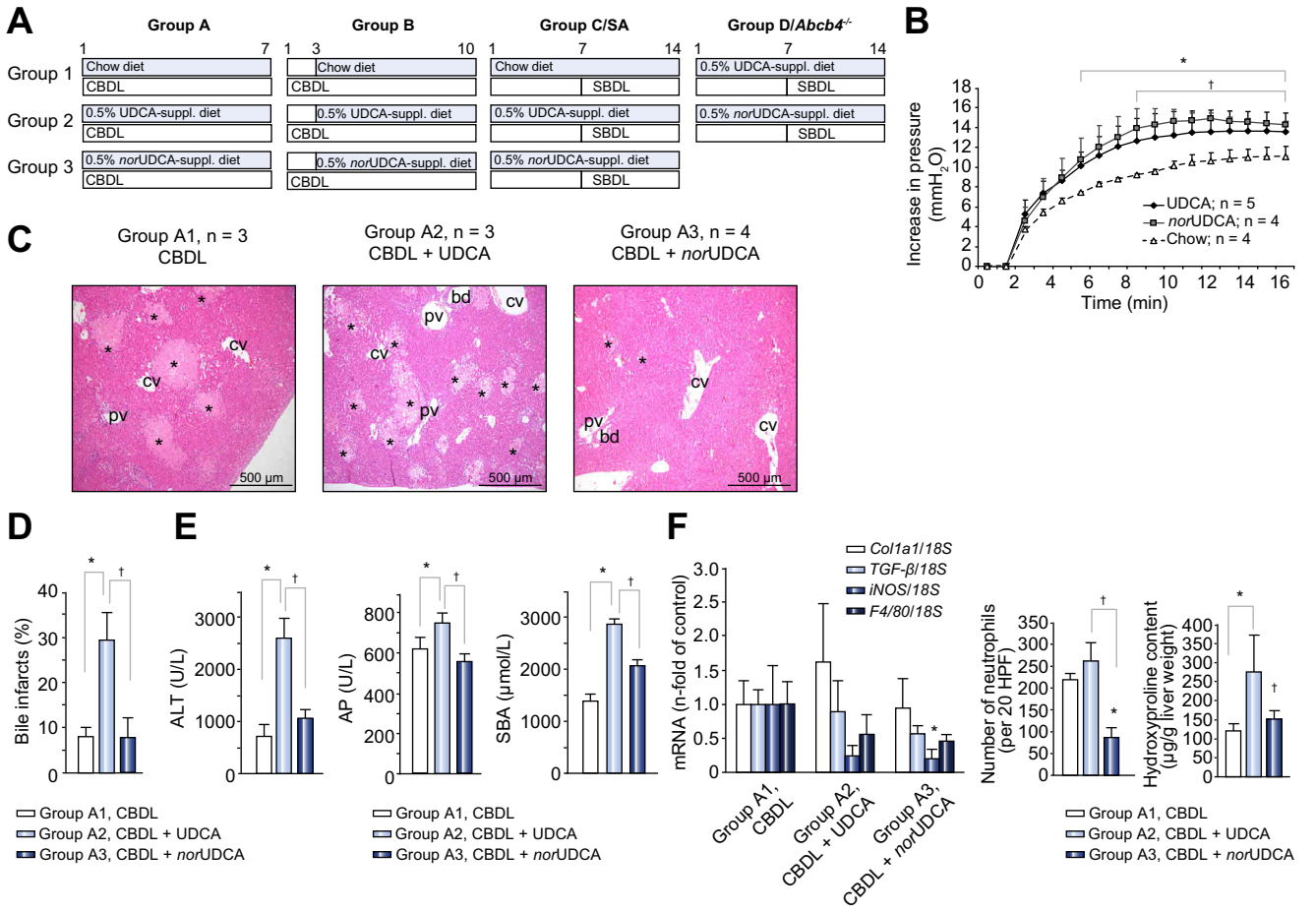

Fig. 1. UDCA but not *nor*UDCA aggravates liver injury in bile duct ligated mice. (A) Experimental design: mice were fed either 0.5% UDCA, 0.5% *nor*UDCA, or chow diet, subjected to common bile duct ligation (CBDL) or selective bile duct ligation (SBDL), and harvested 7, 10 or 14 days later. Mice received bile acid-supplemented diet either at time of CBDL (Group A) or 3 days past CBDL (Group B). SBDL was performed 7 days after start of bile acid feeding in Swiss albino (Group C) and *Abcb4*$^{-/-}$ mice (Group D). (B) Manometric tracing in UDCA (black lozenges), *nor*UDCA (gray squares) and chow-fed mice (open triangles). Biliary pressure in *nor*UDCA-fed mice is higher without reaching statistical significance compared to the UDCA-fed group. Note that significant differences compared to chow-fed mice were reached at an earlier time point in *nor*UDCA-fed mice. p <0.05: *CBDL *vs.* CBDL + UDCA. †CBDL *vs.* CBDL + *nor*UDCA. (C) Liver histology of CBDL mice fed chow (Group A1), 0.5% UDCA- (Group A2) and 0.5% *nor*UDCA-supplemented diet (Group A3) for 7 days. While UDCA increases the number and size of bile infarcts (indicated by asterisks) in CBDL mice, *nor*UDCA-feeding results in a comparable or even slightly improved histological picture. (D) Morphometric analysis reveals a significantly higher amount of bile infarcts in UDCA-fed compared to *nor*UDCA- and chow-fed CBDL mice. (E) While UDCA significantly increases serum AP levels compared to chow- and *nor*UDCA-fed CBDL mice, *nor*UDCA-feeding leads to a slight AP reduction compared to chow-fed CBDL mice. Both UDCA and *nor*UDCA significantly increase serum bile acid (SBA) levels compared to chow-fed CBDL mice. (F) Significantly reduced *iNOS* mRNA, hepatic neutrophil count, and hepatic hxydroxyporine levels in *nor*UDCA-treated CBDL mice. Values are mean ± SD from 3–4 animals per group. p <0.05: *CBDL *vs.* CBDL + UDCA or CBDL + *nor*UDCA. †CBDL + UDCA *vs.* CBDL + *nor*UDCA. (C) Original magnification 100×. bd, bile duct; cv, central vein; pv, portal vein.

injury, with aggravation of bile infarcts, when started immediately following CBDL (Fig. 1C and D) as indicated by significantly increased serum ALT, AP, and SBA levels (Fig. 1E). In contrast, compared to the UDCA group, *nor*UDCA-fed CBDL mice showed significantly reduced liver injury in this experimental setting (Fig. 1C and D) and ALT levels were not significantly elevated compared to chow-fed controls (Fig. 1E). In addition, *nor*UDCA led to a significant downregulation of *iNOS* mRNA, reduced the number of infiltrating neutrophils, and lowered hepatic hydroxyproline content compared to chow-fed controls (Fig. 1F). To more closely model the clinical scenario of initiating bile acid treatment in a situation with an already 'fixed' obstructive component, both drugs were started 3 days post CBDL (Group B). Liver injury was again significantly aggravated in the UDCA-fed group and significantly lower in *nor*UDCA-fed CBDL mice when compared to the UDCA group (Fig. 2A and B), but interestingly less pronounced when compared to the findings in Group A. However, only serum AP levels were significantly reduced in the *nor*UDCA-fed group, whereas differences in ALT levels did not reach statistical significance (Fig. 2C), indicating potential differences in the dynamics of ALT changes following CBDL between experimental Groups A and B. Again, *nor*UDCA feeding resulted in a significant downregulation of hepatic *iNOS* mRNA levels compared to chow-fed mice, and significantly reduced the number of infiltrating neutrophils and the content of hydroxyproline (Fig. 2D). Thus taken together, *nor*UDCA – in strong contrast to UDCA – did not significantly increase liver injury in CBDL mice. Bile infarct areas were even smaller compared to chow-fed CBDL controls; however, these changes did not reach statistical significance.

Table 1. Bile flow and composition in mice under chow diet, 0.5% UDCA-, and 0.5% *nor*UDCA-supplemented diet for 3 days. Values are mean ± SD from 3–5 animals per group. p <0.05: *Chow *vs.* UDCA or *nor*UDCA; †UDCA *vs.* *nor*UDCA.

Variable	Chow n = 3	UDCA n = 4	*nor*UDCA n = 5
pH	7.6 ± 0.1	7.7 ± 0.1*	7.8 ± 0.1*
Bicarbonate concentration (mmol/L)	34.3 ± 2.2	52.1 ± 9.10*	72.3 ± 2.4*†
Bile acid concentration (mmol/L)	15.0 ± 2.6	29.2 ± 9.5*	17.4 ± 3.1†
Phospholipid concentration (mmol/L)	4.1 ± 0.5	2.8 ± 0.2*	1.2 ± 0.2*†
Cholesterol concentration (mmol/L)	0.4 ± 0.1	0.6 ± 0.2	0.2 ± 0.1†
Bile flow (µl/gLW/min)	2.8 ± 0.4	5.4 ± 0.9*	6.3 ± 0.7*
Bicarbonate output (nmol/gLW/min)	96.8 ± 17.7	278.3 ± 64.4*	457.7 ± 66.3*†
Bile acid output (nmol/gLW/min)	42.6 ± 11.3	151.3 ± 27.9*	108.8 ± 15.7*†
Phospholipid output (nmol/gLW/min)	11.5 ± 2.8	14.7 ± 2.3	7.2 ± 1.0*†
Cholesterol output (nmol/gLW/min)	1.2 ± 0.4	3.2 ± 0.6*	1.2 ± 0.7†

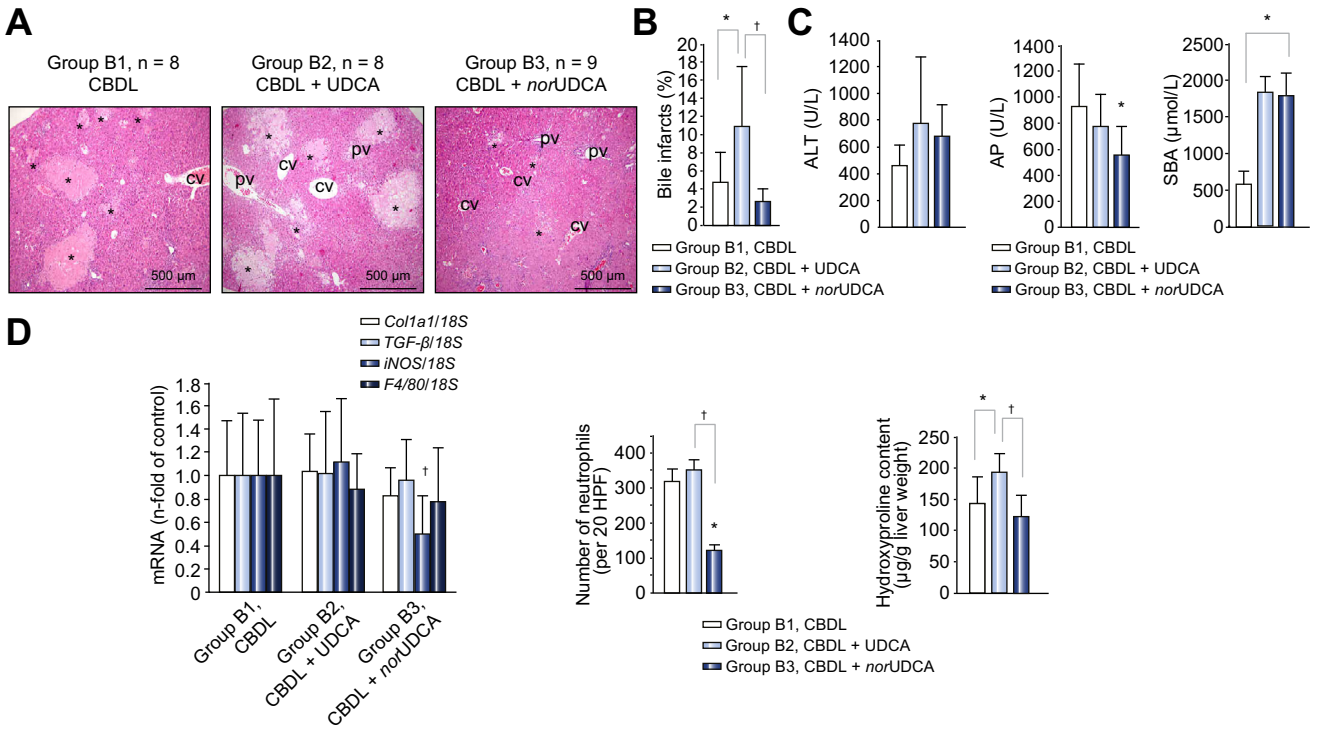

Fig. 2. *nor*UDCA ameliorates liver injury in CBDL mice. (A) H&E stained liver sections from mice fed chow (CBDL, Group B1), 0.5% UDCA- (CBDL + UDCA, Group B2) and 0.5% *nor*UDCA-supplemented diet (CBDL + *nor*UDCA, Group B3) 3 days past CBDL. Note the higher number and size of bile infarcts (indicated by asterisks) in UDCA-fed mice. (B) Morphometric analysis shows a significantly higher amount of bile infarcts in UDCA-fed compared to *nor*UDCA-fed CBDL mice. (C) Comparable ALT values between all experimental groups, while UDCA leads to an increase in AP levels, *nor*UDCA significantly reduces AP levels compared to CBDL chow-fed mice. Both UDCA and *nor*UDCA significantly increase serum bile acid (SBA) levels in CBDL mice compared to CBDL chow-fed mice. (D) Significantly reduced *iNOS* mRNA levels, hepatic neutrophil count, and hepatic hydrocyproline levels in the *nor*UDCA group. Values are mean ± SD from 8–9 animals per group. p <0.05: *CBDL *vs.* CBDL + UDCA or CBDL + *nor*UDCA. † CBDL + UDCA *vs.* CBDL + *nor*UDCA. (A) Original magnification 100×. bd, bile duct; cv, central vein; pv, portal vein.

Only UDCA but not norUDCA induces bile infarcts in SBDL mice

Next, we compared the differential effects of UDCA and *nor*UDCA in SBDL mice to model the clinical situation of a main bile duct stricture in cholangiopathies using wild type (Fig. 3, Group C) and *Abcb4*$^{-/-}$ mice (Fig. 4, Group D). UDCA feeding induced pronounced bile infarcts in the ligated lobes of SBDL mice, visible already at the macroscopic level in WT mice (Fig. 3A upper panel) and further confirmed histologically, and quantified by morphometric analysis (Fig. 3A lower panel, Fig. 3B). In contrast, chow-fed SBDL mice showed an enlarged ligated liver lobe (Fig. 3A upper panel) with ductular reaction in the ligated lobes and some small bile infarcts (Fig. 3A lower panel, Fig. 3B). Notably and in contrast, *nor*UDCA-fed SBDL mice showed no bile infarcts on a macroscopic and microscopic level but ductular reaction and pronounced periductal edema in the ligated lobes, indicating sufficient SBDL

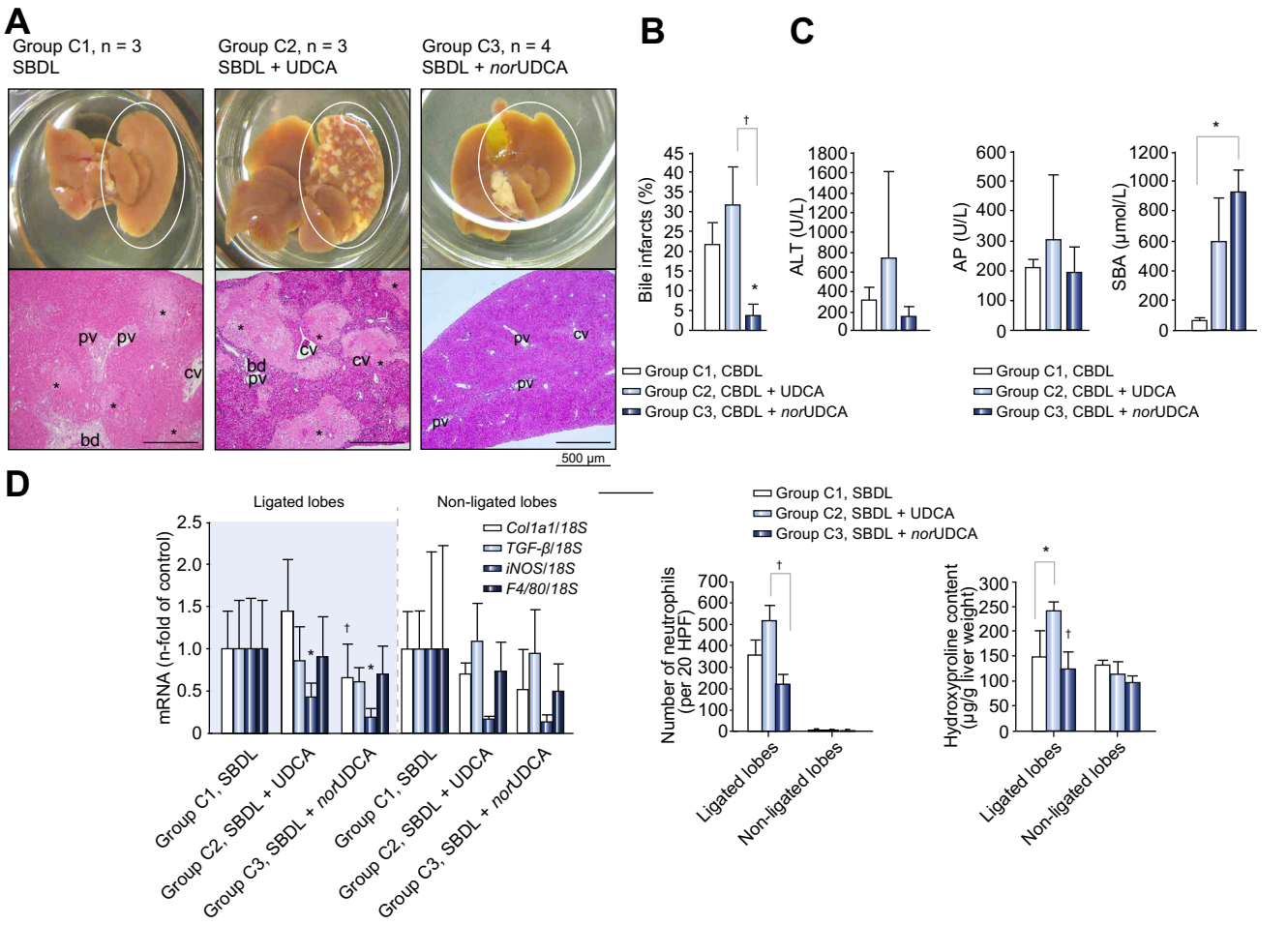

Fig. 3. UDCA but not *nor*UDCA aggravates bile infarcts of ligated lobes in SBDL mice. (A) Macroscopic appearance of the hepatic *facies visceralis* and H&E stained liver sections in chow-fed SBDL liver (SBDL, Group C1), UDCA-fed SBDL liver (SBDL + UDCA, Group C2), and *nor*UDCA-fed SBDL liver (SBDL + *nor*UDCA, Group C3). Note that only the UDCA-fed mouse shows bile infarcts (white circles) in SBDL lobes. Note periductal edema and ductular reaction under all experimental situations. Notably, only the UDCA-fed SBDL liver shows bile infarcts (indicated by asterisks). (B) Morphometric analysis shows a significantly higher amount of bile infarcts in UDCA-fed mice. (C) Differences in serum ALT and PP values did not reach statistical significance. Both UDCA and *nor*UDCA cause a significant increase in serum bile acid (SBA) in SBDL mice. p <0.05: *CBDL *vs.* CBDL + UDCA or CBDL + *nor*UDCA. †CBDL + UDCA *vs.* CBDL + *nor*UDCA. (D) Significant reduced neutrophil count and hepatic hydroxyproline content in the *nor*UDCA group. Values are mean ± SD from 3–4 animals per group. p <0.05: *SBDL *vs.* SBDL + UDCA or SBDL + *nor*UDCA. †SBDL + UDCA *vs.* SBDL + *nor*UDCA. (A lower panel) original magnification 100×. bd, bile duct; cv, central vein; pv, portal vein.

(Fig. 3). Significantly induced ductular reaction in the ligated lobes was additionally confirmed by immunohistochemistry using a cholangiocyte-specific anti-K19 antibody and quantified by Western blotting (Supplementary Fig. 1). Comparing UDCA- and *nor*-UDCA-fed SBDL *Abcb4*$^{-/-}$ mice (Group D) to model the situation of the development of a main bile duct stricture in SC under bile acid treatment, we observed significantly reduced liver injury in *nor*UDCA-fed *Abcb4*$^{-/-}$ mice compared to UDCA-treated mice as quantified by morphometric analysis of bile infarct areas (Fig. 4A and B). In addition, serum AP levels were significantly reduced in *nor*UDCA-fed SBDL *Abcb4*$^{-/-}$ mice; indicating reduced cholestasis and cholangitis (Fig. 4C). Interestingly, in general, bile infarct areas were smaller in this specific experimental group using *Abcb4*$^{-/-}$ mice (Fig. 4A and B) compared to experiments in wild type mice (Groups A–C). In addition, ductular reaction was significantly reduced in *nor*UDCA- compared to UDCA-treated *Abcb4*$^{-/-}$ mice (Fig. 4E and F). Notably, *nor*UDCA reduced liver injury, ductular reaction, inflammation and biliary fibrosis in non-ligated lobes of SBDL *Abcb4*$^{-/-}$ mice, as demonstrated by significantly reduced neutrophil counts, VCAM-1, K19, and α-SMA protein expression levels (Fig. 4D and F). However, differences in hepatic hydroxyproline did not reach statistical significance after 5 days of *nor*UDCA treatment (Fig. 4D).

Taken together, these findings clearly demonstrate superior therapeutic effects of *nor*UDCA over its mother compound UDCA in the used model systems mimicking main bile duct strictures in cholangiopathies.

UDCA induces cellular ATP depletion and cell death at lower doses than norUDCA in HepG2 cells and is significantly more toxic in primary human hepatocytes

Since UDCA, in contrast to *nor*UDCA, forms, at the intracellular level, a coenzyme A thioester, which is finally conjugated with taurine/glycine, using considerable amounts of cellular ATP (Supplementary Ref. 39), we hypothesized that high-dose UDCA

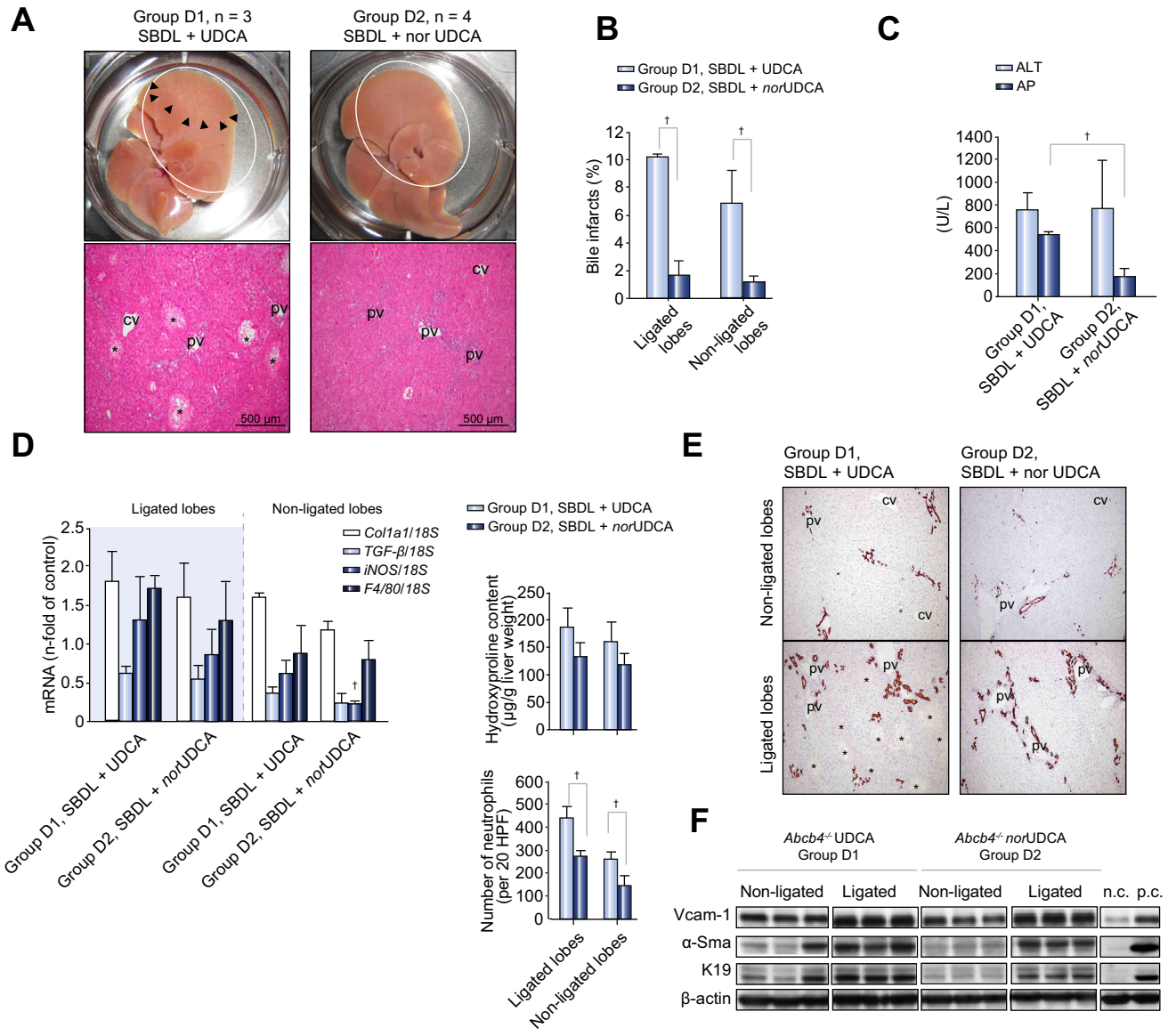

Fig. 4. UDCA but not *nor*UDCA aggravates bile infarcts of ligated lobes in SBDL *Abcb4*$^{-/-}$ mice. (A) Macroscopic liver appearance in the UDCA-fed SBDL liver (SBDL + UDCA, Group D1) and *nor*UDCA-fed SBDL liver of *Abcb4*$^{-/-}$ mice (SBDL + *nor*UDCA, Group D2). Bile infarcts (indicated by black arrowheads) can be detected only in the ligated lobe (white circles) of UDCA-fed mice. Liver histology (H&E staining) reveals periductal edema and ductular reaction under all experimental situations, but only the UDCA-fed SBDL liver shows bile infarcts (indicated by asterisks). (B) Morphometric analysis shows a significant increase in the amount of bile infarcts in the ligated and non-ligated lobes of UDCA- compared to *nor*UDCA-fed *Abcb4*$^{-/-}$ mice under SBDL. (C) UDCA significantly increases serum AP levels compared to *nor*UDCA-fed SBDL *Abcb4*$^{-/-}$ mice whereas ALT levels are comparable. (D) Significantly reduced neutrophil count in the *nor*UDCA groups whereas differences in hepatic hydroxyproline levels did not reach statistical significance. (E) Ductular reaction in ligated lobes of SBDL *Abcb4*$^{-/-}$ mice. Immunohistochemical staining of bile duct epithelial cells by using an anti-K19 antibody. Note that only the ligated lobes (lower panel) show ductular reaction under all experimental conditions. Bile infarcts (indicated by asterisks) are only detected in UDCA-fed SBDL *Abcb4*$^{-/-}$ mice. (F) Western blot analyses of hepatic Vcam-1, α-SMA and K19 protein levels. Note a significant decrease in Vcam-1 and K19 protein levels in the ligated lobes of *nor*UDCA-fed *Abcb4*$^{-/-}$ mice (n.c., negative control, representing loaded liver protein without respective primary antibodies; p.c., positive control, representing liver of untreated *Abcb4*$^{-/-}$ mouse). (A lower panel and D) Original magnification 100×. cv, central vein; pv, portal vein. Values are mean ± SD from 3–4 animals per group. p <0.05: †SBDL + UDCA *vs.* SBDL + *nor*UDCA.

may induce cellular ATP depletion and significantly reduce cell viability in comparison to *nor*UDCA. Indeed, cellular ATP depletion and cell death were significantly increased in UDCA-treated compared to *nor*UDCA-treated HepG2 cells (Supplementary Fig. 2A and B). Most importantly, *nor*UDCA did not affect cell viability in primary human hepatocytes, at doses up to 2000 μM, while UDCA already reduced cell viability significantly at 150 μM after one-hour treatment (Supplementary Fig. 3A and B), indicating that our experimental findings in mouse models and HepG2 cells may be also relevant to the human condition. We also hypothesized that both bile acids have different cytotoxicity to bile duct epithelial cells (BECs), and therefore we tested both bile acids in raising concentrations up to those determined in bile from bile acid-fed mice (Table 1), and observed that UDCA was significantly more toxic than *nor*UDCA (Supplementary Fig. 4).

Discussion

The current study was stimulated by our previous findings with aggravation of bile infarcts in UDCA-treated mice with obstructive jaundice [Supplementary Ref. 31], raising potential safety concerns on the use of even more choleretic *nor*UDCA in human cholangiopathies with an obstructive component. Since we hypothesized that *nor*UDCA would aggravate liver injury in CBDL and SBDL mice due to its potent choleretic effects [23], we designed the present straightforward experiments to directly compare the effects of UDCA and *nor*UDCA on bile formation in mice, in mouse models with clear-cut obstructive cholestasis, and in regard to potential differences in cytotoxicity using *in vitro* systems for hepatocytes. Interestingly, *nor*UDCA did not further aggravate liver injury in CBDL mice and, even more surprisingly, attenuated liver injury in SBDL mice. The combined findings of these experiments argue against our previous assumption that aggravation of bile infarcts in UDCA-fed CBDL mice primarily depends on its choleretic effects, since *nor*UDCA has comparable effects on biliary pressure. Accordingly, several intriguing aspects of the current study should stimulate a fruitful discussion on the different effects of *nor*UDCA and its mother compound UDCA, in particular in regard to the search for the explanation of recently reported detrimental effects of high-dose UDCA in PSC [Supplementary Ref. 43].

There is a persuasive body of experimental evidence for a critical link between bile flow, biliary bile acid composition, biliary pressure on the one hand, and the degree of liver injury, bile infarcts, and fibrosis on the other hand [Supplementary Refs. 30,32,34,41,42]. Our current head-to-head comparison of UDCA- and *nor*UDCA-fed mice revealed comparable choleretic potency and increases in biliary pressure in response to bile duct obstruction in both groups. It is important to note that *nor*UDCA exclusively induced bicarbonate-dependent bile flow, whereas UDCA also significantly induced biliary bile acid concentration and therefore bile acid-dependent bile flow. This led to twofold higher biliary bile acid concentrations in UDCA-fed mice compared to the *nor*UDCA group. Since bile infarcts represent oncotic hepatocyte cell death induced by regurgitated bile, with millimolar concentrations of bile acids, into the parenchyma via disrupted canals of Herring, it is obvious that bile in *nor*UDCA-treated mice is less toxic compared to the UDCA groups. This concept is further supported by our current *in vitro* findings showing significant higher cytotoxicity of UDCA compared to *nor*UDCA when tested on HepG2 cells, Hepa 1.6 cells (not shown), rodent BECs, and most importantly, on primary human hepatocytes (Supplementary Figs. 2–4). UDCA in comparison to *nor*UDCA induced hepatocellular ATP depletion in HepG2 cells already at significant lower doses, which is associated with a significant higher degree of cell death in UDCA-treated cells. This may be related to the fact that UDCA forms a coenzyme A thioester and is finally conjugated with taurine/glycine, a process consuming considerable amounts of cellular ATP. In contrast, with *nor*UDCA, little coenzyme A thioester is formed, as previously shown by Kirkpatrick [Supplementary. Ref. 39]; subsequently, hepatocytes are not depleted from ATP to the extent observed in UDCA-treated cells, which may represent an important mechanism for the observed differences in cytotoxicity. Importantly, UDCA was already toxic at 150 μM concentrations on primary human hepatocytes, whereas *nor*UDCA toxicity occurred earliest with 2000 μM (Supplementary Fig. 3), suggesting a potential human relevance of our experimental findings. Consequently, reduced liver injury in *nor*UDCA-treated mice may origin in (i) lower biliary bile acid concentrations (approximately the half of UDCA-treated mice), (ii) differences in bile acid-induced ATP depletion and associated cytotoxicity between both molecules, and (iii) higher biliary bicarbonate concentration in the *nor*UDCA-treated groups. The higher biliary bicarbonate concentration in *nor*UDCA-fed animals may create a protective milieu in a situation with complete bile duct obstruction, as demonstrated also by the beneficial effects in SBDL mice. Taken together, the combined findings of these experiments argue for the concept that the origin and cause for choleresis of a bile acid or derivative may critically determine its potential therapeutic efficacy but also toxicity in obstructive cholestasis.

The current study demonstrates substantial differences in the amount of bile infarcts between the different experimental groups in response to UDCA treatment with the highest degree of bile infarcts in concomitantly UDCA-fed CBDL mice (Group A2). In contrast, bile infarct areas were much smaller when UDCA was started 3 days past CBDL and in UDCA-fed SBDL *Abcb4*$^{-/-}$ mice (Groups B2, D1). This may be related to an already induced alternative excretory bile acid excretion pathway, in 3-day CBDL and also in *Abcb4*$^{-/-}$ mice. Alternatively, this may probably origin in periductal fibrosis in these experimental conditions. Since a periductal fibrotic shield, representing a kind of wound healing of bile ducts, may protect the liver parenchyma in cholangiopathies where leaking bile is engaged, a solely or targeted antifibrotic strategy, without cure of the underlying cause or initiating factor for periductal fibrosis, could consequently even worsen liver injury in this difficult-to-manage group of patients.

Our experimental results obtained in mice, tested a 30-fold higher *nor*UDCA dose compared to that used in an on-going phase II clinical trial, and suggest that *nor*UDCA should not have a high potency in increasing liver injury in cholestatic liver disease with obstructive component. However, it has to be kept in mind that *nor*UDCA compared to UDCA is superior in regard to its choleretic potency in humans. In addition, bicarbonate-dependent bile flow – primarily stimulated by *nor*UDCA – is even more important according to quantity and pathophysiological relevance in humans compared to rodents. Consequently, *nor*UDCA should be tested very cautiously in patients suffering from cholangiopathies with obstructive component. On the other hand, *nor*UDCA-induced bicarbonate-rich choleresis could embody a protective mechanism in cholangiopathies via flushing bile ducts primarily with a hydrophilic bile acid and water, leading to substantial dilution of endogenous biliary secreted bile acids, creating a less toxic milieu within the bile ducts. Moreover, *nor*UDCA was significantly less toxic to murine BECs compared to UDCA. Whether *nor*UDCA will open a protective "bicarbonate umbrella" for bile ducts will have to be determined [Supplementary Ref. 44] and this concept may need to be expanded by bicarbonate dilution of bile.

Taken together, our findings clearly demonstrate significant assets of *nor*UDCA in regard to biliary physiology and superior therapeutic efficacy in different models for cholangiopathies with obstructive component in comparison to UDCA, which argues for the notion that induction of biliary bicarbonate secretion should be a promising therapeutic concept for cholangiopathies.

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Conflict of interest

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2013.01.026.

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