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Protective Effects of 6-Ethyl Chenodeoxycholic Acid, A Farnesoid X receptor (FXR) Ligand, In Estrogen Induced Cholestasis

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Running title

FXR and cholestasis

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Abbreviations used in this paper.

ALT, alkaline phosphatase; CA, cholic acid; CDCA, chenodeoxycholic acid; LCA, lithocholic acid; 6-ECDCA, 6 ethyl-CDCA, FXR, farnesoid X receptor; T-βMCA, tauro β-muricholic acid; $E_2 17\alpha$, ethynilestrodiol; SHP, small heterodimer

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partner; cyp7α1, cholesterol 7α-hydroxylase; cyp8β1, oxysterol 12β-hydroxylase; Bsep, bile salt export pump; *ntcp*, Na^+/taurocholate cotransporting peptide; *mdr1*, multidrug resistance protein-1; *mdr3*, multidrug resistance protein-3; *mrp2*, multidrug resistance-associated protein-2;

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The farnesoid X receptor (FXR), an endogenous sensor for bile acids, regulates a program of genes involved in bile acid biosynthesis, conjugation, and transport. Cholestatic liver diseases are a group of immunologically and genetically mediated disorders in which accumulation of endogenous bile acids play a role in the disease progression and symptoms. Here we describe the effect of 6-ethyl chenodexyxholic acid (6-ECDCA) a semi-synthetic bile acid derivative and potent FXR ligand in a model of cholestasis induced by 5-day administration of 17α-ethynylestradiol ($E_2$17α) to rats. The exposure of rat hepatocytes to 1 μM 6-ECDCA caused a 3-5 fold induction of small heterodimer partner (*Shp*) and bile salt export pump (*bsep)* mRNA and 70-80% reduction of *cyp7a1* (cholesterol 7α-hydroxylase), *cyp8b1* (oxysterol 12β-hydroxylase) and *ntcp* (Na^+/taurocholate cotransporting peptide). In vivo administration of 6-ECDCA protects against cholestasis induced by $E_2$17α. Thus, 6-ECDCA, reverted bile flow impairment induced by $E_2$17α, reduced secretion of cholic acid and deoxycholic acid, but increased muricholic acid and CDCA secretion. In vivo administration of 6-ECDCA increased liver expression of *Shp*, *bsep, mrp2* and *mdr2* while reduce *cyp7a1 and cyp8b1* and *ntcp* mRNA. These changes were reproduced by GW4064, a synthetic FXR ligand. In conclusion, by demonstrating that 6-ECDCA protects against $E_2$17α cholestasis, our data support the notion that development of potent FXR ligands might represent a new approach for the treatment of cholestastic disorders.

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Introduction

Cholestasis results in systemic and intrahepatic retention of potentially toxic bile acids that causes liver injury, ultimately leading to biliary fibrosis and cirrhosis (Trauner et al., 1998). Estrogens are well known to cause intrahepatic cholestasis in susceptible women during pregnancy, administration of oral contraceptives, and postmenopausal replacement therapy (Bossard et al., 1993; Huang et al., 2000; Kullak-Ublick et al., 2004; Stieger et al., 2000; Vore, 1987). Cholestasis induced by administration of estrogen to rodents, a model used to gain mechanistic insights in this condition, has been linked to a reduction of activity and/or expression of several transporters including: 1) the ATP-binding cassette (ABC) bile salt export pump (*bsep*) (Bossard et al., 1993; Stieger et al., 2000); 2) *mdr1a/1b*, the two multidrug resistance protein-1 (MDR1) isoforms expressed in rodents (Bossard et al., 1993); and 3) the multidrug resistance-associated protein-2 (*mrp2*) (Huang et al., 2000). In addition, estrogen-induced cholestasis associates with a reduced activity of the Na+/taurocholate co-transporting polypeptide (*ntcp*) (Bossard et al., 1993; Simon et al., 1996) and a reduction of endogenous bile acid synthesis (Simon et al., 1996).

While physiological actions of estrogens are mediated by the classic signaling pathway in which an estrogen receptor binds to an estrogen response element in the promoter of a gene, recent studies (Lai et al., 2003) have demonstrated that estrogens increase the liver expression of the short heterodimer partner (*Shp*), an atypical nuclear receptor that lacks a DNA-

binding domain (DBD) and is a target for farnesoid-X-receptor (*FXR*) ligands. *Shp* is known to repress the activity of many nuclear hormone receptors *in vitro* and is critically involved in regulating bile acid synthesis. Whether *Shp* activation mediates changes in bile acid synthesis in a rodent model of cholestasis induced by estrogen administration is still unclear.

Bile acids act as signaling molecules that regulate their own biosynthesis and transport by binding to and activating the *FXR* (Forman et al., 1995; Makishima et al. 1999; Parks et al., 1999; Seol et al., 1995; Wang et al. 1999;Wang et al., 2002). *FXR* alters the transcription of target genes by binding DNA sequences composed of two inverted repeats separated by one nucleotide (IR-1) as a heterodimer with the 9-*cis*-retinoic acid (9-*cis*-RA) receptor (*RXR*α (Forman et al., 1995; Seol et al., 1995). Activation of *FXR* initiates the transcription of a cohort of genes that function to decrease the concentration of bile acids within the hepatocyte. Specifically, FXR induces the expression of the genes encoding *BSEP* (Sinal et al., 2000; Ananthanarayanan et al., 2001), multidrug resistance protein 3 (*MDR3*) (Holst et al., 2003; Kerr et al., 2002; Wang et al., 2002), and MRP2. In addition, FXR activation leads to a feedback repression of *cyp7a1* and cyp8b1 (del Castillo-Olivares and Gill, 2001; Goodwin et al., 2000*)*. These genes encode for the cholesterol 7α-hydroxylase and the sterol 12α-hydroxylase, both of which are central to the synthesis of bile acids from cholesterol (Goodwin et al., 2000; del Castillo-Olivares and Gill, 2001). In rodents, the *FXR*-dependent suppression of *cyp7a1* is mediated by S*hp* (Goodwin et al., 2000; del Castillo-Olivares and Gill, 2001) which interacts with *LRH-1* (liver receptor homolog-1), a known positive

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regulator of *cyp7a1* and represses its transcriptional activity (Goodwin et al., 2000; del Castillo-Olivares and Gill, 2001; Kerr et al., 2002; Lu et al. 2000; Wang et al., 2002).

We have previously reported that modification of CDCA by addition of an ethyl group in its position 6 results in a semi-synthetic bile acid, the 6-ECDCA, which has potent *FXR* agonist activity (Pellicciari et al., 2002; Costantino et al., 2003; Mi et al., 2003; Fiorucci et al., 2004). The resolution of the crystal structure of the FXR ligand binding domain complexed with 6-ECDCA and the coactivator peptide Src-1 (Mi et al., 2003) demonstrates that in the presence of 6-ECDCA, helix 12, the activation function 2 domain of the receptor, adopts the agonist conformation and stabilizes the binding of the coactivator peptide (Costantino et al., 2003). This would provide a mechanism for enhanced binding of co-activators through intermolecular contacts between their LXXLL sequences, explaining the enhanced potency of 6-ECDCA in comparison with the natural ligand.

Because activation of *FXR* might have the potential to protect the liver in cholestatic diseases (Liu et al., 2003), we have designed a study to investigate whether administration of 6-ECDCA, protects against cholestasis development induced by estrogen and to investigate the molecular targets involved in this effect.

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Materials and Methods

Materials

The synthesis of 6-ECDCA is described elsewhere (22). CDCA, 17α-ethynylestradiol ($E_2$17α) were from the Sigma Chemical Co. (St. Louis, MO).

In vitro studies

Effect of 6-ECDCA on rat FXR-regulated genes

Rat hepatocytes were prepared as described elsewhere by *in situ* collagenase perfusion through the hepatic portal vein (Fiorucci et al., 2002). After isolation cells were suspended in DMEM medium containing 10% FBS, 1 nM insulin, 0.15 mg/ml methionine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cell viability was >86% (trypan blue dye exclusion test). Hepatocytes were then plated in matrix/matrigel coated culture plates at a density of 1 x 10^5 cells per dish in a 95% air and 5% CO_2. Cells were then incubated with 1 μM 6-ECDCA for 24 hours and total RNA extracted for quantitive (q)RT-PCR analysis.

In vivo studies

Adult male Wistar rats weighing 300 to 350 g were used throughout the studies. Before the experiments, the animals were maintained on standard chow and water *ad libitum,* and housed in a temperature- (21°C - 23°C) and humidity- (45%-50%) controlled room under a 12-hour light, 12-hour dark cycle.

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All studies were approved by the Animal Study Committee of the University of Perugia.

Surgical procedures and bile flow measurement

For bile flow measurement, animals were anesthetized with a single dose of sodium pentobarbital (50 mg/kg body wt, intraperitoneally), and maintained under this condition throughout the experiment. After catheterization of the jugular vein and carotid arthery using a PE-50 polyethylene tubing (Intramedic, Clay Adams, Parsippany, NJ), a middle abdominal incision was made, and the common bile duct was also cannulated (PE-10, Intramedic, Clay Adams). Body temperature was maintained at 37.0°C to 38.5°C with a warming lamp to prevent hypothermic alterations of bile flow. Bile flow was determined by gravimetry, assuming a density of the bile of 1.0 g/ml. The biliary excretion rate was calculated as the product between bile flow and biliary concentration. The bile was assayed for both total and individual bile salts by high pressure liquid chromatography (HPLC) as previously described (Setchell et al., 1997). Total bile acid concentrations were derived by the sum of individual bile acids (Russell and Setchel, 1992).

Estrogen-induced cholestasis

Animals were randomly divided into experimental groups (8-12 rats/group) as described in Table 1. For bile collection, surgical procedures were made on the sixth day (i.e., 1 day after the administration of the last dose of $E_2 17\alpha$). Bile collection started between 9:00 and 11:00 AM to minimize influence of circadian

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variations. Bile was collected at 15-minute intervals for 120 min and bile flow determined gravimetrically (Kern et al., 1977; Stieger et al., 1994; Koopen et al., 1999). At the end of the study the livers were perfused for 30 s with saline and the major lobe was removed. Liver samples were gently frozen in liquid nitrogen and preserved at –80°C until used for qRT-PCR studies.

Routine serum biochemistry and bile acid measurements

Serum samples were stored at –70°C until analysis of aspartate aminotransferase (AST), bilirubin, alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GT) by routine clinical chemistry testing performed on a Hitachi 717 automatic analyzer .

qRT-PCR

After the rats had been killed, livers were removed and immediately snap-frozen on liquid nitrogen and stored at –80°C until used. Total RNA was isolated using TRIzol reagent (Life Technologies, Milan, Italy) as previously described (Fiorucci et al., 2004). Quantification of the expression rat genes was performed by qRT-PCR) using the following sense and antisense primers: *ntcp*: 5'-gcatgatgccactcctcttatac-3' and 5'-tacatagtgtggccttttggact-3'; *bsep*: 5'-aaggcaagaactcgagataccag-3' and 5'-tttcactttcaatgtccaccaac-3'; *cyp7α1*: 5'-ctgcagcgagctttatccac-3' and 5'-cctgggttgctaagggactc-3'; *cyp8β1*: 5'ccctatctctcagtacacatgg3' and 5'gaccataaggaggacaaaggtct3'; *Shp*: 5'-cctggagcagccctcgt-3' and 5'-aacactgtatgcaaaccgagga-3'; *FXR*: 5'-tggactcatacagcaaacagaga-3' and 5'-gtctgaaaccctggaagtctttt-3'; *mrp3*:

5’tcagcatcctcatcaggtttatt and 5’atgatagcagtccgtatcctcaa; *ntc*p: 5’gcatgatgccactcctcttatac3’ and 5’tacatagtgtggcctttggact3’; *Gapdh*: tccgcccctcc gctgatg 3' and 5' cacggaaggccatgccagtga 3'. All PCR primers were designed using software PRIMER3-OUTPUT using published sequence data from the NCBI database. The RNA was reverse-transcribed with Superscript III (Invitrogen) in 20 µl reaction volume using random primers. For RT-PCR, 100 ng template was used in a 25 µl containing 0.3 µM of each primer and 12.5 µl of 2X SYBR Green PCR Master mix (Bio-Rad). All reactions were performed in triplicate and the thermal cycling conditions were as follows: 2 min at 95°C, followed by 50 cycles of 95°C for 10 sec, and 60°C for 30 sec in an iCycler iQ instrument (Biorad, Hercules, CA). The mean value of the replicates for each sample was calculated and expressed as the cycle threshold (C_T: cycle number at which each PCR reaction reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (.C_T) between the C_T value of the sample for the target gene and the mean C_T value of that sample for the endogenous control (*Gapdh*). Each gene is expressed as a ratio between control (untreated) and treated rats.

Statistical analysis

Data reported are the mean ± SE of the number of experiments indicated. The statistical analysis was carried out using a GraphPad Prism 3, GraphPad Software Inc. (San Diego, CA). The analysis of variance (ANOVA) and the Student's t-test for paired data were employed when appropriate.

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Results

6-ECDCA transactivates FXR and modulates FXR regulated genes in hepatocytes

To examine whether 6-ECDCA has the ability to regulate genes involved in bile acid synthesis and transport, rat hepatocytes were incubated with a maximally effective concentration of 6-ECDCA, 1 μM, and its parent molecule CDCA, 100 μM, for 24 hours and mRNA expression of *Shp, cyp7a1, cyp8b1, bsep, ntcp* and *oatp1* measured by qRT-PCR. As shown Figure 1b, we found that exposure to FXR ligands increases *Shp* and *bsep* mRNA expression by 3-5 fold while reduced *cyp7a1, cyp8b1* and *ntcp* mRNA by 50-70% (n=5; P<0.05 versus control cells). In contrast 6-ECDCA and CDCA failed to modulate *oatp1* gene expression (Figure 1b). To investigate the effect of estrogen on gene involved in bile acid synthesis and transport, rat hepatocytes were exposed to 100 nM $E_2 17\alpha$. As shown in Figure 1c, exposure to $E_2 17\alpha$ increased *Shp* mRNA expression by ≈2 fold (n=5; P<0.05 versus control cells), but failed to inhibit *cyp7α1* and *cyp8β1* and *ntcp* mRNA expression. Thus, in contrast to FXR ligands induction of liver expression of *Shp* by $E_2 17\alpha$ do not translate in inhibition of the *cyp7a1 and cyp8b1.*

6-ECDCA protects against cholestasis induced by $E_2 17\alpha$

Five days administration of $E_2 17\alpha$ slightly decreased body weight (Figure 2*a*) which was parallelled by increased levels of serum ALP, a biochemical marker of cholestastis (n = 6-8 animals; P< 0.05 versus control rats) and bile acids. In contrast, $E_2 17\alpha$ treatment failed to modify either ALT, bilirubin and

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γ-GT levels (not shown). Administration of 6-ECDCA (Figure 2*a-d*) failed to reverse the body weight lost caused by estrogen but improved serum ALP activity (n = 6-8; P<0.05 versus $E_2$17α at 10 mg/kg/day 6-ECDCA). Treating rats with CDCA did not improve either the body weight, serum ALP or bile acid levels (n = 6-8; P>0.05 versus $E_2$17α). Consistent with these biochemical changes, bile flow analysis carried out 24 h after the last dose of 6-ECDCA or CDCA (Figure 3A-C and Table 2) showed an impairment of bile flow that dropped from 68.0 ± 3.4 µl/Kg/min in control rats to 36.9 ± 3.5 µl/Kg/min (-48 ± 5%) in rats treated with 5 mg/kg $E_2$17α (n = 6-8; P<0.01). Administration of 6-ECDCA induced a dose-dependent improvement of cholestatic changes caused by $E_2$17α. At the dose 10 mg/kg/day (Figure 3A and Table 2), it completely reverted cholestasis induced by $E_2$17α (n = 6-8; P < 0.01 versus $E_2$17α). In contrast, no protection was observed in rats treated with 10 mg/kg/day CDCA (Figure 3B and Table 2). Analysis of total bile acid output demonstrates that the decrease in total bile salt output induced by $E_2$17α was primarily accounted for by a decrease in the excretion of TCA (-55% versus control) and TDCA (-71% versus control) and, to a lesser extent, β-MCA (-13% versus control) (Table 2). Administration of 6-ECDCA partially prevented the impairment in total bile acid output caused by $E_2$17α by increasing the relative abundance of β-MCA (not significantly different from animals receiving $E_2$17α alone) and TCDCA (+ 27%; P< 0.05 versus $E_2$17α) and TDCA (+ 74%, P< 0.05 versus $E_2$17α). Despite the significant enrichment in TCDCA and TDCA

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caused by CDCA it failed to protect against bile flow impairment caused by $E_2$17α (n=6-8; P>0.05 versus $E_2$17α).

Confirming the protective role of FXR ligands on this animal model, we found that GW4064 (Figure 1) was also effective in reducing cholestasis as measured by serum ALP. activity (n=8-10; P<0.05 versus $E_2$17α). GW4064 (Figure 3C and Table 2) also attenuated bile flow impairment caused by $E_2$17α (n=8-10; P<0.05 versus $E_2$17α) and increased the relative abundance of β-MCA (+13%; P>0.05 versus $E_2$17α) and TCDCA (+ 55%; P<0.05 versus $E_2$17α) and TDCA (+ 28%, P>0.05 versus $E_2$17α).

As shown in Figure 4, 5-day administration of $E_2$17α slightly increased liver *Shp* mRNA expression and reduced *cyp7a1 and cyp8b1* mRNA by 40-70% (n = 4, P< 0.01 versus control) (Figure 4A-C). Furthermore, $E_2$17α treatment decreased *ntcp* and *oatp1* mRNA expression by ≈30-40% (n = 4, P< 0.01 versus control; Figure 5A and D), but had no effect on *bsep* mRNA levels (n=4, P>0.05 versus control; Figure 5G). Furthermore, $E_2$17α administration associate with ≈20% reduction of *mrp2* mRNA (Figure 5B) while it caused a ≈8 fold increase in *mrp3* gene expression (Figure 5E), and 2-4 fold induction of *mdr1a and mdr1b* (n=4, P<0.05 versus control; Figure 5C and F). Administration of 6-ECDCA resulted in a 3-4 fold increase of *Shp* mRNA expression (n = 4; P< 0.05 versus control, Figure 4C) which associates with a further decrease in *cyp7a1* (n=4, P<0.01 versus control; Figure 4A) and *cyp8b1* (n=4, P<0.01 versus rats administered $E_2$17α; Figure 4B,) mRNA, the latter being reduced to almost undetectable levels. Consistent with

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Shp induction treating rats with 6-ECDCA caused a ≈80% reduction of *ntcp* mRNA (n=4; P<0.05 versus control; Figure 5A), and increased *bsep*, *mrp2* and *mdr2* mRNA by 2-4 fold; n-4, P<0.05 versus control; Figures 5B, E and H) Administering rats with 6-ECDCA reduced *mdr1a* mRNA expression while had no effect on induction of *mrp3,* and *mdr1b* caused by $E_2$17β (Figure 5C,. In contrast to 6-ECDCA, CDCA failed to upregulate *Shp* mRNA expression over the effect of $E_2$17α (n=4, P<0.05 in comparison with control) resulting in a comparable inhibition of *cyp7a1 and cyp8b1* mRNA expression in the liver (n=4; P<0.05 versus 6-ECDCA) Figure 5). CDCA was also less effective than its derivative in regulating *ntcp, bsep, mrp2* and *mdr2* mRNA (n=4, P>0.05 versus $E_2$17α). As shown in Figure 5, similarly to 6-ECDCA, CDCA, had no effect on either *mrp3, mdr1a* and *mdr1b* mRNA when compared with $E_2$17α (n=4; P>0.05).

Confirming the protective role of *FXR* against cholestasis development, treating rats with GW4064 increased *Shp* expression by ≈4 fold and reduced liver *cyp7a1* and *cyp8b1* mRNA by 60-70% (P<0.05 versus $E_2$17α). GW4064 administration increased *bsep, mrp2* and *mdr2* mRNA expression by 2-4 fold (n=4; P<0.05 versus control), while similarly to 6-ECDCA it had no effect on *mrp3, mdr1a* and *mdr1b* (P>0.05 versus $E_2$17α). Finally, none of the treatment was effective in modulating *oatp1* mRNA (n=4; P>0.05 versus $E_2$17α).

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Discussion

In vitro studies in human and rodent hepatocytes have provided evidence that *FXR* regulates bile acid synthesis by modulating the activity of *CYP7A1* the rate limiting enzyme in the pathway involved in conversion of cholesterol into bile acids (Chiang, 2002). In the normal liver LXRα, a nuclear receptor for oxysterols, acts as a cholesterol sensor and up-regulates the expression of *CYP7A1* in response to increased levels of its ligands (Goodwin et al., 2000; Lu et al., 2000). *CYP7A1*'s enzymatic activity leads to the production of bile acids that bind to *FXR* and activate *SHP* (Goodwin et al., 2000; Lu et al., 2000; Peet et al., 1988). This regulatory circuit is supported by the finding that *FXR* null mice have reduced levels of *Shp* and lack the ability to feedback repress *cyp7a1* in response to cholic acid (CA) feeding (Sinal et al., 2000). We now demonstrated that this pathway operates also during cholestasis. Indeed, 5-day administration of $E_2 17\alpha$ was associated with increased expression of *Shp* (Lai et al., 2003) and significant reduction (-40%) of *cyp7a1* mRNA together with a complete inhibition of *cyp8b1* mRNA expression.

In vitro exposure of rat hepatocytes to estrogen increases *Shp* mRNA but, in contrast to the FXR ligands induction of *Shp* caused by $E_2 17\alpha$ failed to repress *cyp7a1* mRNA expression, suggesting that estrogen do not directly regulate this gene expression (Lai et al., 2003). The basis for the lack of repression of *cyp7a1* and *cyp8b1* despite the induction of *Shp* by estrogen in rodents remains unclear. In SHP deficient mice, CA still represses *cyp7a1* expression via alternative mechanisms suggesting that SHP induction by itself

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might not be sufficient to mediate repression of *cyp7a1* and *cyp8b1* without additional signaling pathways (Goodwin et al, 2000; Lu et al., 2000). FXR activation could provide additional signals such as production of a SHP ligand with the ability to confer SHP repression upon *CYP7A1* and *CYP8B1* promoters.

$E_2$17α administration associated with a number of changes in the expression of basolateral and canalicular transporters (Figure 6A). Thus 5-day administration of $E_2$17α caused a ≈30-40% reduction of *ntcp* and *oatp1* mRNA (Huang et al., 2000; Stieger et al., 1994; Trauner et al., 1998; Kullak-Ublick et al, 2003). Because *ntcp* is an important component of the sinusoidal systems that import bile acid into hepatocytes, and its expression is regulated by FXR-SHP regulatory cascade (Kullak-Ublick et al, 2003) a reduction of this transporter is likely to develop as an adaptation to represent cholestasis.

Our qRT-PCR analysis also demonstrates that the level of *mrp3, mdr1a* and *mdr1b* were increased in rats administered with $E_2$17α. These canalicular transporters mediate efflux of xenobiotics and basolateral excretion of anion conjugates from hepatocytes. *Mdr1a* and *mdr1b* expression is regulated by PXR, the pregnane x receptor (Goodwin et al., 2003; Maglich et al., 2002). Since PXR is also activated, among other ligands, by bile acids (Goodwin et al., 2003; Kast et al., 2002), induction of *mdr1a* and *mdr1b* is likely to represent an adaptive change that protects the liver by providing a mechanism for clearance of toxic constituents from hepatocytes.

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Bile acids bind and activate FXR with CDCA being the most active ligand (Forman et al., 1995; Makishima et al., 1999; Parks et al., 1999; Wang et al. 1999). Activation of FXR by endogenous ligand, however requires micromolar concentrations, suggesting that development of potent FXR agonists, active in the nanomolar range, could help to activate this sensor mechanism providing a mechanism for clearance of toxic constituents from hepatocytes. One potential risk of FXR agonists, however, might result from the feedback inhibition of bile acid synthesis, a mechanism that might cause a further impairment of cholestasis by reducing the biliary flow driven by bile salts. Our results however, demonstrate that administration of potent FXR ligands protects against cholestasis development despite it completely inhibited *cyp7a1* and *cyp8b1* mRNA expression. The main explanation for this effect is that while CYP7A1 catalyse the key, rate-limiting step of the *classical,* major pathway of the overall bile salt synthesis, bile acids can also be generated through an alternative pathway that involves the mitochondrial sterol 27-hydroxylase (*CYP27A1*) (Russell and Setchel, 1992). In contrast to the classical pathway that leads to synthesis of CA and deoxycholic acid (DCA), *CYP27A1* catalyses the first step in the *alternative* pathway which produces selectively CDCA and the bile salts derived from its further hepatic and/or intestinal conversion, *i.e.,* MCA (Russell and Setchel, 1992). C*YP27A1* is not regulated by *FXR*, suggesting that bile acid synthesis through this pathway could be maintained even in the presence of *FXR* ligands. Consistent with this view, the analysis of individual bile acids in the bile of $E_2$17α-treated rats (Table 2) demonstrated a relative enrichment of the bile salt pool by Tβ-MCA

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and TCDCA (i.e. bile acids generated through the alternative pathway) together with a decrease of CA and its secondary bile salt, DCA (Kern et al., 1977; Stieger et al., 1994; Koopen et al., 1999).

In the present study we have demonstrated that a synthetic an FXR ligand protects against development of cholestasis induced by estrogen administration to rats. At the dose of 10 mg/kg 6-ECDCA profoundly inhibited *of ntcp* expression and caused, along with a 3-fold increase of Shp, a 2-3 folds increase in *bsep, mrp2* and 5-fold increase in *mdr2* mRNA. Since these basolateral *(ntcp*) and canalicular (*bsep, mrp2 and mdr2*) transporters are directly regulated by FXR (Denson et al., 2001) and their repression/induction is not observed or is only partially observed (*ntcp*) in rats treated with $E_2 17\alpha$ alone, their regulation is likely to play a mechanistic role in the anti-cholestatic effect of 6-ECDCA (Kullak-Ublick et al, 2003). Supporting this view, it has been shown that the *cis* and *trans*-inhibition of *bsep* is the main determinant of impaired bile flow in this model (Kullak-Ublick et al, 2003). *BSEP* and *MRP2* are ATP binding cassette proteins that transport bile acids and other organic anions across the canalicular membrane (Kullak-Ublick et al, 2003). Canalicular transport of bile acids is a rate-limiting step in hepatic excretion of bile acids and *bsep* and *mrp2* are critically involved in this process. Mutation of *BSEP* and *MRP2* genes in humans associated with cholestatic disorders (Trauner et al., 1999; Kullak-Ublick et al, 2003). Thus inactivating mutation of *BSEP* gene give rise to the type 2 of progressive familial intrahepatic cholestasis (Strautnieks et al., 1998), while mutations of MRP2 give rise to Dubin-Johnson syndrome (Paulusma et al., 1997), a disease

characterized by high serum bilirubin due to the inability of liver to excrete this metabolite into the bile. Induction of BSEP and MRP2 expression is under the control of FXR, and their modulation in response to CA feeding is lost in FXR null mice (Sinal et al., 2000). Similarly to *BSEP*, *MRP2* has an IR1 responsive element in the promoter region (Kullak-Ublick et al., 2004) that directly binds the *FXR/RXR* heterodimer and is activated by CDCA and 6-ECDCA in vitro. The demonstration that *bsep* expression is unchanged in rats rendered cholestatic by estrogen administration, but is induced by 6-ECDCA and GW4064, however, indicates that both positive and negative mediators are generated in the liver of cholestatic animals that might affect the expression of this canalicular transporter. Interestingly, the cholestatic bile acid litocholic acid (LCA) inhibits the BSEP promoter and strongly decreases BSEP expression induced by CDCA in human hepatocytes through antagonism of FXR activity (Yu et al., 2002). Although LCA is a weak FXR agonist it might function as a partial antagonist in vivo displacing more potent endogenous ligands from their binding site. In contrast to CDCA, however, *bsep* induction caused by 6-ECDCA can not be reverted *in vitro* by LCA (Fiorucci et al, unpublished), suggesting that potent FXR ligands might overcome the effect of LCA In vivo.

In addition to *bsep* and *mrp2*, we have shown that 6-ECDCA administration increases *mdr2*/MDR3 expression by ≈5 fold. Induction of *mdr2*/MDR3 by the FXR ligand GW4064 has recently been described in cholestasis induced by bile duct ligation and α-naphtyl-isothiocyanate (Liu et al., 2003). This canalicular transporter, the phospholipid flippase, mediates

biliary secretion of phospholipids and cholesterol, thus its induction by FXR ligand appears to be mechanistically involved in the anti-cholestatic effect induced by FXR ligands.

Since 6-ECDCA is CDCA derivative, there is a possibility that its effects are due to its conversion into CDCA. However, this seems not to be the case. Indeed we found that T-CDCA concentrations did not increased in rats treated with 6-ECDCA, while a significant enrichment was observed in animals treated with CDCA alone, suggesting that 6-ECDCA is not converted to CDCA during its liver metabolism.

In conclusion we have provided evidence that induction of the canalicular transporters *bsep, mrp2* and *mdr2*/MDR3 and repression of bile acid biosynthesis mediate anti-cholestatic effects of 6-ECDCA. By demonstrating that 6-ECDCA protects against cholestasis induced in rat by estrogen administration our data support the notion that development of potent FXR agonists might be beneficial in the treatment of cholestastic disorders.

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Footnotes

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Figure legends

Figure 1. 6-ECDCA is a potent and selective activator of FXR. (Panel A) Chemical structure of 6-ECDCA. (Panel B) 6-ECDCA increases the expression of FXR regulated genes in rat hepatocytes. Cells were incubated with 6-ECDCA (1 μM) for 24 hours and gene expression monitored by qRT-PCR. Data are mean ± SE of 4 experiments. *P<0.05 versus untreated cells. (Panel C). Exposure of rat hepatocytes to $E_2$17α increases *Shp* mRNA but fails to repress *cyp7a1 and cyp8b1* mRNA expression. Data are mean ± SE of 4 experiments. *P<0.05 versus untreated cells.

Figure 2. Five-day administration of 6-ECDCA, and GW4064 failed to protect against weight loss (panel A) and changes in body weight/liver weight ratio (panel B) but reduced serum ALT (panel C) in rats treated with 5 mg/kg $E_2$17α. In contrast to GW4064, 6-ECDCA and CDCA increases plasma bile acid concentrations (panel D). Data are mean ± SE of 6-8 rats per group. * P< 0.05 versus control; **P < 0.05 versus $E_2$17α alone.

Figure 3. Panel A-C. Effects of 5-day administration of 6-ECDCA, CDCA and GW4064 on bile flow in administered $E_2$17α. Bile flow was measured as described in Materials and Methods after 5 days of administration of $E_2$17α alone or in combination with 6-ECDCA, CDCA or GW4064. Data are mean ±

SE of 6-8 rats per group. * P < 0.05 versus control; **P < 0.05 versus $E_2$17α alone.

Figure 4. Panel A-C**.** Expression of *cyp7a1, cyp8b1* and *shp* mRNA following 5-day administration of 5 mg/kg $E_2$17α alone or in combination with 6-ECDCA (10 mg/kg), CDCA (10 mg/kg) or GW4064 (20 mg/kg). Data are mean ± SE of 4 rats per group each assay carried out in triplicate.

Figure 5. Panel A-H. Effect of 5-day administration of 6-ECDCA (10 mg/kg), CDCA(10 mg/kg), and GW4064 (30 mg/kg) on the expression of basolateral and canalicular transporters in rats administered 5 mg/kg $E_2$17α for 5 days Data are mean ± SE of 4 rats per group each assay was carried out in triplicate. *P<0.05 versus control.** P<0.05 versus $E_2$17α alone.

Figure 6. Summary of changes of basolateral and canalicular transporters in rats administered $E_2$17α alone (panel A) or in combination with 6-ECDCA (panel B). Administration of 6-ECDCA increased the expression of *bsep, mrp2* and *mdr2* providing a mechanism for excretion of toxic metabolites from hepatocytes. The grey squares and large arrows denote genes that are modified by FXR ligands beyond the effect of $E_2$17α.

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Table 1. Protocol treatments

Treatment regimen	Number of rats	Duration of Treatment (days)
Control	12	5
17α-ethynylestradiol ($E_2$17α) 5 mg/kg	8	5
$E_2$17α+ 6-ECDCA 1 mg/kg	8	5
$E_2$17α+ 6-ECDCA 5 mg/kg	8	5
$E_2$17α+ 6-ECDCA 10 mg/kg	8	5
$E_2$17α+ CDCA 10 mg/kg	8	5
$E_2$17α+ GW4064 30 mg/kg	8	5

All drugs were administered intraperitoneally from day 1 to day 5.

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Table 2. Effect of natural and synthetic FXR ligands on bile acid output in $E_2 17\alpha$ induced cholestasis.

	Control	$E_2 17\alpha$ 5 mg/kg/ day ip	$E_2 17\alpha$ + 6-EDCA 10 mg/kg/ day ip	$E_2 17\alpha$ + CDCA 10 mg/kg/day ip	$E_2 17\alpha$ + GW4064 30 mg/kg/ day i.p
Bile flow (ul/kg min)	68.0 ± 6.0	33.3 ± 3.0*	66.0 ± 4.1**	39.0 ± 5 .0*	71.0 ± 4.1**
Total bile acid output (µg/Kg min)	187.5 ± 31.2	129.7 ± 35.3*	152.6 ± 28.3**	298.5 ± 239.9**	161.3 ± 17.3**
Individual bile salts					
Tβ-MCA	87.1 ± 11.6 (46.5%)	75.9 ± 16.4 (58.8%)	76.1 ± 14.1 (49.6%)	70.7 ± 54.9 (23.5%)	91.3 ± 10.8 (56,6%)
TCA	77.1 ± 14.0 (41.1%)	42.3 ± 13.3* (32,0%)	33.1 ± 5.7* (23,7%)	63.0 ± 55.6 (21.3%)	47.0 ± 8.2 (29.1%)
TCDCA	8.7 ± 2.3 (4,6%)	7.3 ± 3.3 (5.6%)	9.3 ± 0.9 (6.4%)	101.1 ± 92.1* 34.2%)	14.1 ± 1.4 (8.7%)
TDCA	14.7 ± 3.3 (7.8%)	4.2 ± 2.3* (3.4%)	7.6 ± 1.7* (4.9%)	63.7 ± 37.3* (21.1%)	7.9 ± 1.4 (5.6%)
T6ECDCA	ND	ND	26.5 ± 5.9* (17.4%)	ND	91.3 ± 10.8 (56.6%)

Number in parenthesis indicate the percent of each individual bile acid
ND= not detected.
Data are mean ± SD of 8-12 rats / group.
*P<0.05 versus control
** P<0.05 versus $E_2 17\alpha$

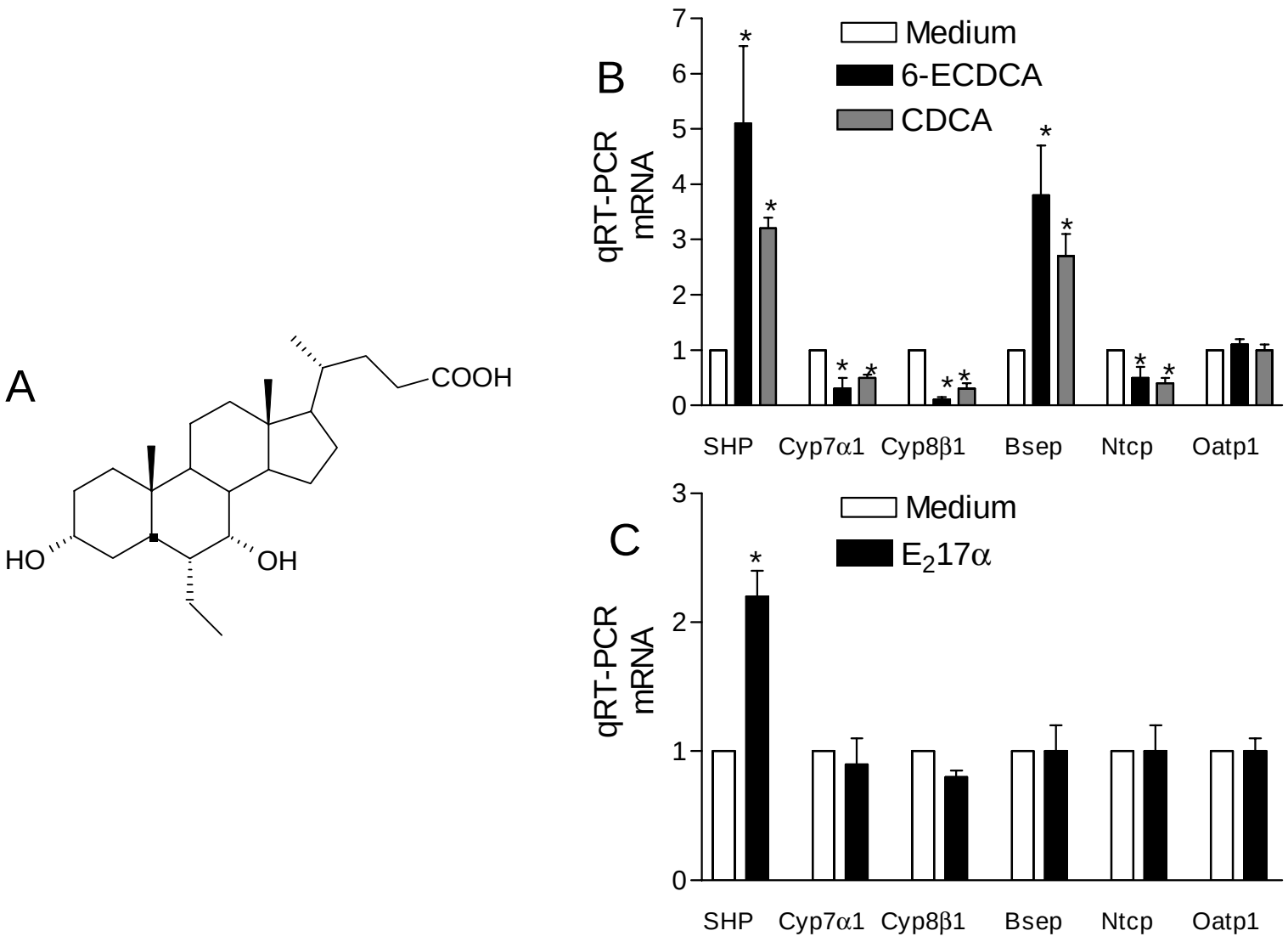


Figure 1

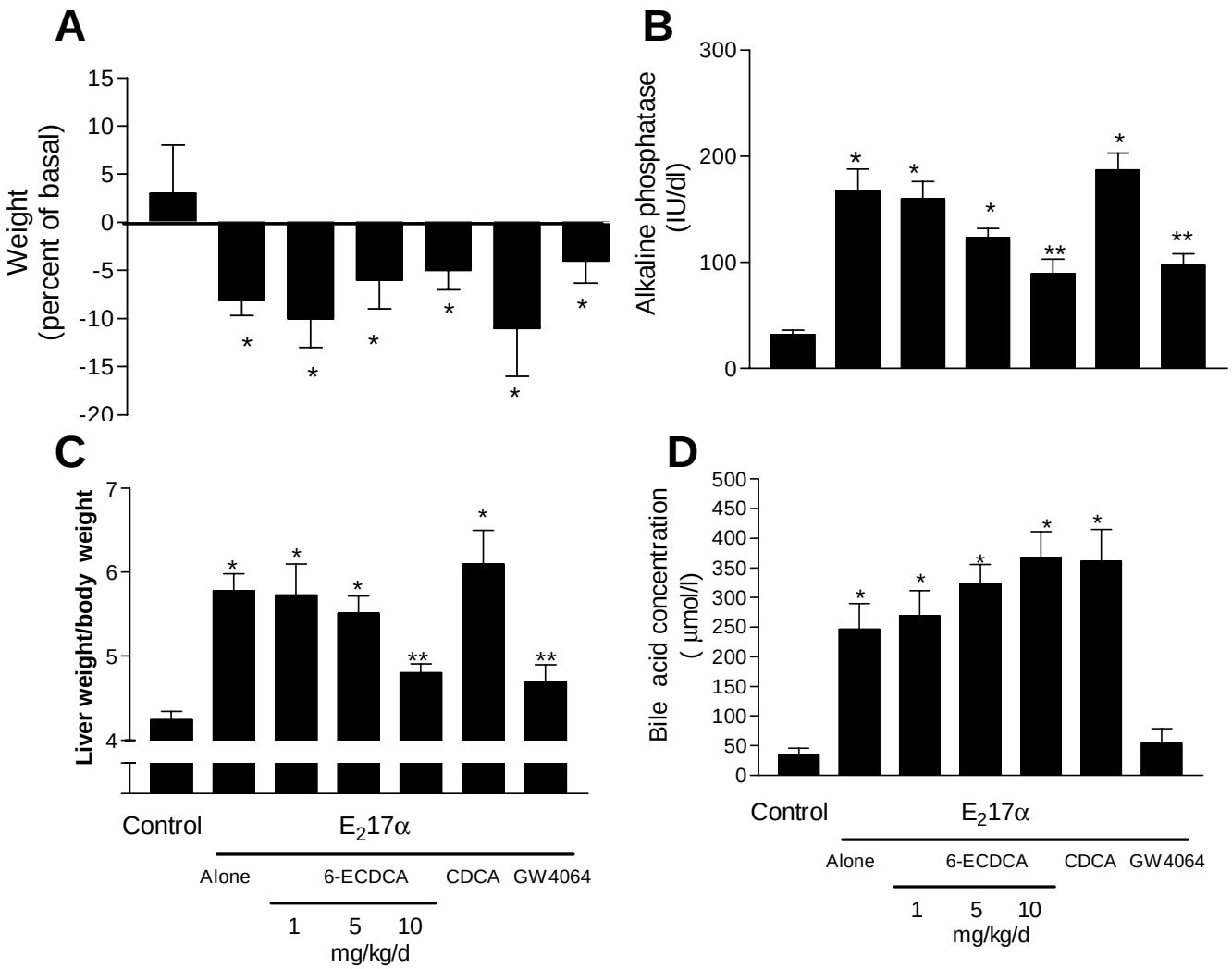
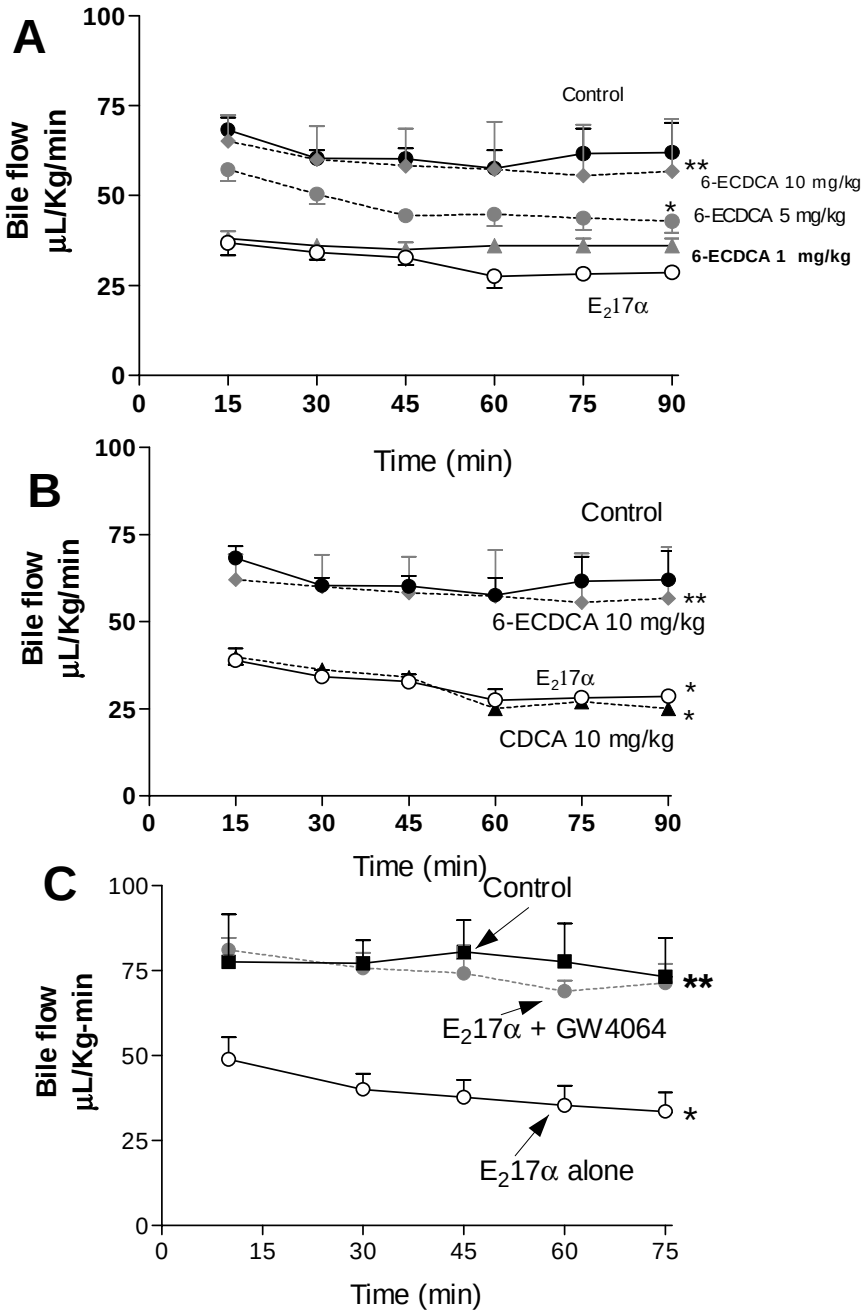


Figure 2



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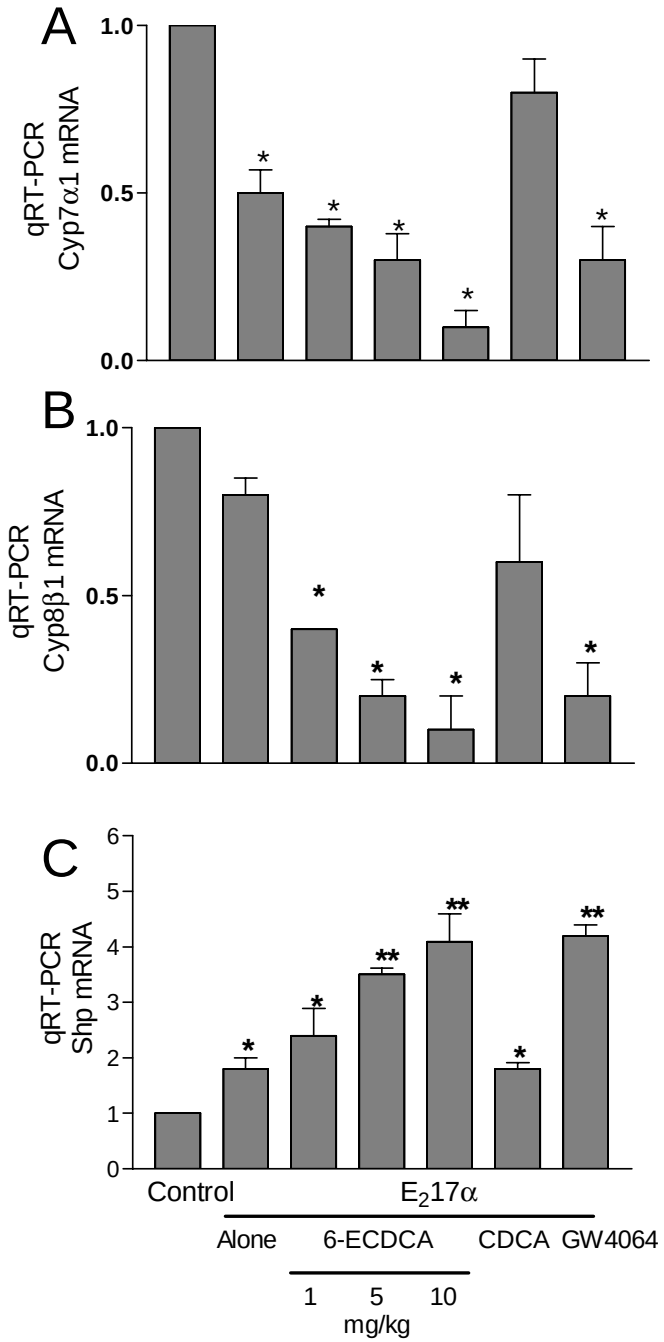
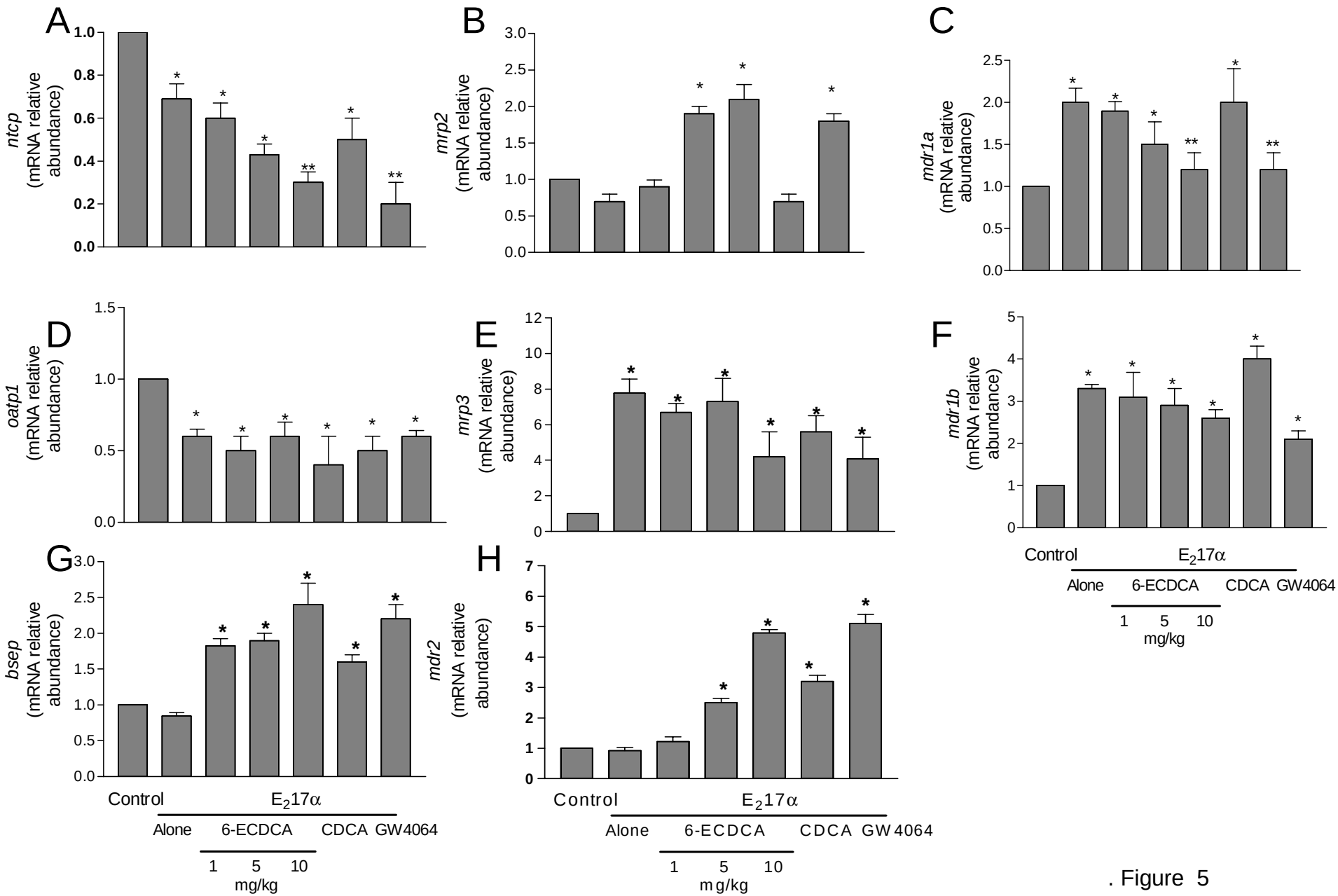


Figure 4



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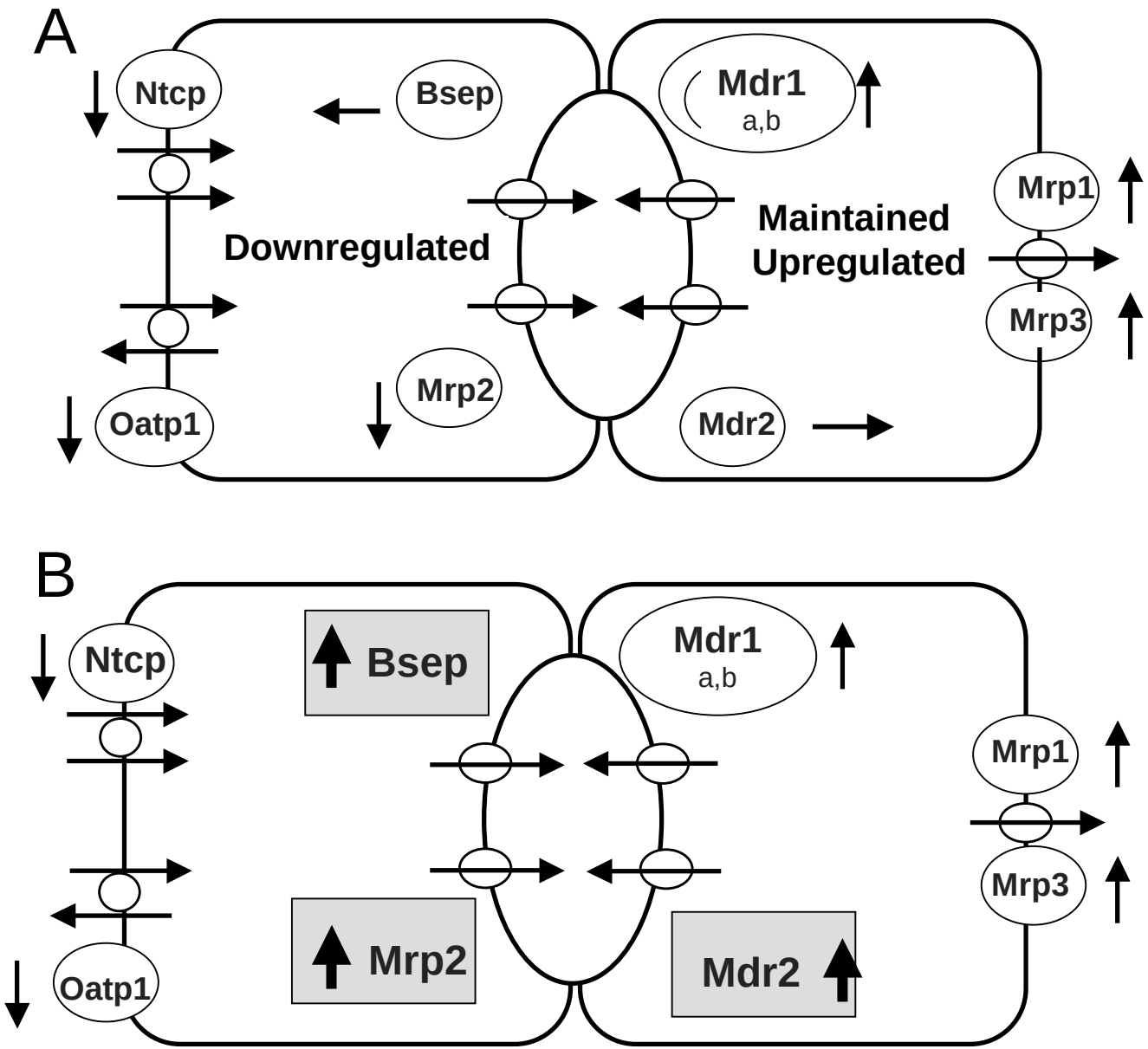

Figure 6