Abstract

Bile acid accumulation in liver with cholangiolar neoplastic lesions may occur before cholestasis is clinically detected. Whether this favors intrahepatic cholangiocarcinoma development has been investigated in this study. The E. coli RecA gene promoter was cloned upstream from Luc2 to detect in vitro direct genotoxic ability by activation of SOS genes. This assay demonstrated that bile acids were not able to induce DNA damage. The genotoxic effect of the DNA-damaging agent cisplatin was neither enhanced nor hindered by the hepatotoxic and hepatoprotective glycochenodeoxycholic and glycocholic acids, respectively. In contrast, thioacetamide metabolites, but not thioacetamide itself, induced DNA damage. Thus, thioacetamide was used to induce liver cancer in rats, which resulted in visible tumors after 30 weeks. The effect of bile acid accumulation on initial carcinogenesis phase (8 weeks) was investigated in bile duct ligated (BDL) animals. Serum bile acid measurement and determination of liver-specific healthy and tumor markers revealed that early thioacetamide treatment induced hypercholanemia together with upregulation of the tumor marker Neu in bile ducts, which were enhanced by BDL. Bile acid accumulation was associated with increased expression of interleukin (IL)-6 and downregulation of farnesoid X receptor (FXR). Bile duct proliferation and apoptosis activation, with inverse pattern (BDL > thioacetamide + BDL >> thioacetamide vs. thioacetamide + BDL > BDL), were observed. In conclusion, intrahepatic accumulation of bile acids does not induce carcinogenesis directly but facilitates a cocarcinogenic effect due to stimulation of bile duct proliferation, enhanced inflammation, and reduction in FXR-dependent chemoprotection.

Implications: This study reveals that bile acids foster cocarcinogenic events that impact cholangiocarcinoma.

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Introduction

The silent symptoms and the lack of specific markers account for a frequent late diagnosis of intrahepatic cholangiocarcinoma, which contributes to a bad prognosis of these patients because tumor progression is already advanced and liver resection is usually not recommended. In addition, cholangiocarcinoma has a poor response to alternative therapeutic options, such as radiotherapy and chemotherapy (1, 2).

Although in many cases the etiology of cholangiocarcinoma remains unknown, some risk factors, such as primary sclerosing cholangitis, hepatolithiasis, parasitic infections, choledochal cysts, and carcinogen exposure, have been associated with its development (1). All these conditions share the characteristic of a certain degree of liver injury due to chronic cholestasis and inflammation. Indeed, cholangiocarcinoma is often associated with chronic cholestasis and it is well known that exposure to high concentrations of bile acids might be associated with an increased incidence of gastrointestinal tumors (3). However, the role of bile acids in cholangiocarcinogenesis remains poorly understood. There is evidence of the relationship, probably through the interaction with the nuclear receptor FXR (farnesoid X receptor) or the membrane-bound receptor TGR5, between bile acids and the proliferation and survival of biliary epithelial cells (4). Moreover, several studies in animals support the relationship between cholestasis and cholangiocarcinoma progression (5, 6). On the other hand, FXR, whose expression is decreased in cholangiocarcinoma (7), is involved in liver cells chemoprotection against genotoxic compounds (8).

To better understand the biology of intrahepatic cholangiocarcinoma, several experimental models have been...
developed. Some of them are based on the treatment with thioacetamide, a thiono-sulfur–containing compound, which in rodents causes acute liver injury after a single dose (9), but cirrhosis and cholangiocarcinoma development after chronic administration (10–12). Thioacetamide has been shown to require metabolic activation by hepatic cytochrome P450 CYP2E1 to give thioacetamide sulfoxide (TASO) and subsequently the highly reactive sulfodioxide (TASO2) (13), which covalently binds to cellular macromolecules and accounts for thioacetamide toxicity.

In this study, the cholangiocarcinogenic effect of thioacetamide, alone or in combination with bile acid retention induced by bile duct ligation (BDL), has been investigated. Moreover, the ability of bile acids to either induce DNA damage directly or to enhance or prevent the effect of genotoxic agents was determined.

Materials and Methods
Animals and experimental groups
Male Wistar rats (280–300 g; University of Salamanca Animal House, Salamanca, Spain), fed on standard rodent chow (Panlab) and water ad libitum, and maintained on 12-hour cycles of light and dark, were divided into four groups: (i) control, (ii) thioacetamide (Sigma-Aldrich Quimica), (iii) BDL, and (iv) thioacetamide + BDL. All protocols were approved by the University of Salamanca Ethical Committee. BDL was carried out as previously described (14). In the thioacetamide + BDL group, thioacetamide administration (0.05% in the drinking water) started the day after BDL. For comparative purposes some animals received thioacetamide for 30 weeks, the time needed for the development of cholangiocarcinomas. Samples were collected from sodium pentobarbital (50 mg/kg body weight, intraperitoneally) anesthetized animals.

Small liver samples or dissected tumors were immediately placed in the RNAlater RNA stabilization reagent (Life Technologies). Additional samples were immersed in liquid nitrogen and stored at −80°C until used for Western blot analysis or histologic analysis. Blood was collected from the inferior cava vein and the serum obtained was stored at −20°C for further use.

Rat hepatocytes
Hepatocytes were isolated from male Wistar rats (220–250 g) as reported previously (15). To obtain conditioned medium containing thioacetamide metabolites, primary cultured hepatocytes were incubated for 24 hours with medium containing different concentrations (5–75 μmol/L) of thioacetamide. Then, cells were trypsinized and resuspended in 100 μL water to obtain cell lysates, which were immediately frozen in liquid nitrogen and stored at −80°C until use.

Cloning procedures, bacterial cultures, and genotoxicity assays
A 110-bp sequence located in the 5′-flanking region of the RecA gene, previously identified as the proximal RecA promoter (RecApr; GenBank accession number X55552), was selected for cloning using genomic DNA from E. coli. Multisite Gateway cloning (Life Technologies) was used to obtain the expression vector containing the firefly luciferase gene (Luc2; Supplementary Table S1). All plasmids underwent gel electrophoresis–based sequencing.

E. coli cells (BL21-DE3 strain) were transformed with the RecApr–Luc2 plasmid and cultured in Luria-Bertani broth medium supplemented with ampicillin (100 μg/mL). Fractions (5 mL) of exponential-phase culture grown to OD600 = 0.3 at 37°C were distributed into ventilated test tubes containing cisplatin, thioacetamide, or bile acids at the desired concentration. All compounds were from Sigma-Aldrich Quimica. Cisplatin and lithocholic acid (LCA) were dissolved in dimethyl sulfoxide (≤0.2% final concentration), thioacetamide, deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid (GCDCA), and glycocholate (GUDCA) were dissolved in water, and rat hepatocyte lysates in culture medium. The bacteria were incubated with the compounds at 37°C with shaking for 2.5 hours. The promoter luciferase activity was measured with the Steady-Glo Luciferase Assay System (Promega) in a LAS-4000 image reader (FujiFilm; TDJ).

Measurement of steady-state mRNA levels
Total RNA from rat liver samples was isolated according to a previously described protocol (14). Retrotranscription was carried out using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). Real-time quantitative PCR (RT-qPCR) was performed using AmpliTaq Gold polymerase (Applied Biosystems) in an ABI Prism 7300 Sequence Detection System (Applied Biosystems; Supplementary Table S2). The mRNA levels of each target gene were normalized with the expression levels of β-actin mRNA. Detection of amplified products was carried out using SYBR Green I. Total RNA from control liver was used as a calibrator. Expression levels were calculated as $2^{-\Delta\Delta C_t}$, where $\Delta C_t$ was the difference of $C_t$ between the target gene and the normalizer in each sample. This was used to calculate $\Delta\Delta C_t$ as the difference of this value between the control RNA and experimental groups.

Apoptosis assays
Apoptosis was evaluated by measuring caspase-3 and -8 activities using Ac-DEVD-AMC and Ac-IETD-AFC (Enzo Life Sciences), as previously reported (16), which were normalized by protein concentrations measured using bovine serum albumin as standard. The mRNA abundances of the Bax-α proapoptotic gene and the Bcl-2 antiapoptotic gene were determined by RT-qPCR.

Western blot analyses
Immunoblotting analyses of liver lysates were carried out in 7.5% to 10% SDS–PAGE, loading 10 μg of protein per lane. Primary antibodies were diluted in PBS–Tween (Supplementary Table S3). Immunoreactive protein bands were visualized by an enhanced chemiluminescent (ECL) detection system (Amersham Pharmacia Biotech) after incubation with appropriate secondary antibodies (IgG–HRP-linked).
Histologic analyses

Of note, 5 μm thick rat liver cryosections (3 pieces from different lobes) were fixed with neutral buffered formalin for 20 minutes and stained with hematoxylin/eosin. Immunostaining was performed on cryosections air-dried and fixed in cold methanol. Primary antibodies were diluted in 2% fetal calf serum in PBS (Supplementary Table S3) and secondary anti-mouse Alexa-488 (Invitrogen) 1:1,000. Nuclei were stained with Dapi (10 μmol/L). Confocal laser-scanning immunofluorescence microscopy was performed using a Zeiss LSM 510 apparatus.

Biochemical and statistical analyses

Routine biochemical parameters were determined in serum as previously described (14). Total serum bile acid concentrations were assayed by an enzymatic/fluorimetric method (14, 17). Data are presented as means ± SEM. After performing an ANOVA test, the Bonferroni method of multiple-range testing was used to calculate the statistical significance of differences among groups.

Results

In vitro genotoxicity test

Luciferase activity in bacteria transformed with the recombinant RecApr–Luc2 plasmid was measured after their incubation with cisplatin, used here as a typical DNA-damaging agent, for 2.5 hours. This time was chosen based on previous studies on the time-course of promoter activity versus bacterial growth (data not shown). A concentration-dependent stimulation of RecApr activity at nontoxic cisplatin concentrations was observed (Supplementary Fig. S1A and S1B). To confirm the cisplatin-induced activation of the bacterial SOS response, RecA expression was measured by RT-qPCR. This revealed a 2-fold increase in RecA mRNA levels when the bacteria were incubated with 50 μmol/L cisplatin (data not shown).

Using this approach, the effect of bile acids at nontoxic concentrations for these bacteria was investigated. The results from one of the concentration-response determination using DCA are shown in Supplementary Fig. S1C and S1D. Similar experiments were carried out with CDCA, GCDCA, and LCA (Fig. 1A–C). None of these bile acids were able to induce an increase in RecApr–Luc2 activity. Because thioacetamide metabolites are believed to be involved in genotoxicity, accounting for the carcinogenic effect of thioacetamide treatment, both pure thioacetamide and thioacetamide metabolites contained in the lysate of hepatocytes previously incubated with thioacetamide were evaluated (Fig. 1D and E). Conditioned medium, but not pure thioacetamide, was able to enhance RecApr–Luc2 activity. Finally, the ability of a potentially hepatotoxic bile acid, such as GUDCA, and a hepatoprotective bile acid such as GUDCA, to enhance or reduce, respectively, DNA damage induced by a genotoxic agent such as cisplatin, was investigated. Neither GCDCA nor GUDCA were able to modify the ability of cisplatin to enhance RecApr–Luc2 activity (Fig. 1F).

In vivo cholangiocarcinoma induction model

The protocol used here to induce cholangiocarcinoma caused a significant reduction in body weight (Supplementary Fig. S2A). In animals treated with thioacetamide for 8 weeks, this loss resulted in 40% lower body weight than controls of the same age (Supplementary Fig. S2A). On the other hand, as previously described (14), 8-week BDL slightly reduced weight gain. Body weight was only 15% lower than in controls of the same age. However, BDL induced higher mortality (50%) than thioacetamide (Supplementary Fig. S2B), which precludes longer experimental protocols using BDL. In thioacetamide + BDL group, body weight was lower than in thioacetamide group and the mortality was similar to that found in BDL group. Tumors were visible after thioacetamide administration for 30 weeks (Supplementary Fig. S2C). The histology revealed a cavernous aspect typical of cholangiocellular adenomas, which were adjacent to invasive intestinal-type and mucin-producing cholangiocarcinoma (Supplementary Fig. S2D). In animals of 8-week thioacetamide group the macroscopic aspect of the liver was normal (data not shown). However, in comparison with control livers (Supplementary Fig. S2E) the histologic analysis revealed areas of bile duct proliferation and leukocytic infiltration with important interindividual variability among animals about the extension of the damage (Supplementary Fig. S2F and S2G). Bile duct proliferation was more evident in BDL group (Supplementary Fig. S2H and S2I). As previously described (14), 8-week BDL induced a severe disorganization of the liver parenchyma (Supplementary Fig. S2H and S2I). In the present study, ≈25% of the parenchyma was replaced by bile duct–like structures surrounded by fibrotic stroma. Leukocytic infiltration and nodules of degenerated tissue rich in inflammatory cells were also observed as from 4 weeks after BDL (14). Smaller areas of bile duct proliferation together with areas of cell degeneration and fibrosis (Supplementary Fig. S2J), and bile accumulation (Supplementary Fig. S2K) were observed in thioacetamide + BDL group.

Serum markers

The hepatotoxic effect of thioacetamide was confirmed by changes in routine serum markers of liver function (Table 1). As expected, markers of cholestasis were increased in serum of BDL groups either with or without thioacetamide treatment. Bile acid concentrations increased 30-fold after 4 weeks of BDL (both in BDL and thioacetamide + BDL groups), and 9-fold (BDL) or 15-fold (thioacetamide + BDL) after 8 weeks. Only gamma-glutamyl transferase (GGT) was higher in the thioacetamide + BDL than in the BDL group. Signs of renal damage such as increased levels of serum urea (Table 1) were observed in the BDL group but, surprisingly, not in thioacetamide + BDL group.

Cell proliferation and differentiation

The expression of cholangiocarcinoma markers cytokeratin 7 (CK-7) and claudin-4 (18, 19) increased in parallel in all experimental groups (Fig. 2A and B, respectively). A trend

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toward a progressive increase in both markers over time was observed in the BDL group. The change was significantly ($P < 0.05$) less in thioacetamide + BDL than in BDL group. Consistent with the histologic results, the administration of thioacetamide alone for 8 weeks induced a mild increase in the expression of $CK-7$ and $claudin-4$, whereas well-established cholangiocarcinoma presented elevated levels of both markers (Fig. 2A and 2B). These changes in mRNA abundance were consistent with changes in protein levels assayed by Western blot analysis (Fig. 2F).

Table 1. Effect of thioacetamide treatment (0.05% in drinking water), BDL, and combination of both (thioacetamide + BDL) on biochemical markers in rat serum of liver and kidney function

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Thioacetamide 4 weeks</th>
<th>Thioacetamide 8 weeks</th>
<th>BDL 4 weeks</th>
<th>BDL 8 weeks</th>
<th>Thioacetamide + BDL 4 weeks</th>
<th>Thioacetamide + BDL 8 weeks</th>
<th>CCA 4 weeks</th>
<th>CCA 8 weeks</th>
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<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>67 ± 7</td>
<td>80 ± 4</td>
<td>94 ± 6</td>
<td>82 ± 6</td>
<td>84 ± 10</td>
<td>75 ± 9</td>
<td>87 ± 10</td>
<td>107 ± 4</td>
<td></td>
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<tr>
<td>AST (U/L)</td>
<td>93 ± 14</td>
<td>283 ± 98$^a$</td>
<td>85 ± 17</td>
<td>173 ± 79</td>
<td>233 ± 22$^a$</td>
<td>195 ± 36$^a$</td>
<td>132 ± 32</td>
<td>137 ± 19</td>
<td></td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>55 ± 8</td>
<td>167 ± 59$^a$</td>
<td>49 ± 5</td>
<td>66 ± 16</td>
<td>54 ± 5</td>
<td>45 ± 7</td>
<td>44 ± 1</td>
<td>42 ± 12</td>
<td></td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>5 ± 0.5</td>
<td>10 ± 1$^a$</td>
<td>14 ± 6$^a$</td>
<td>12 ± 7$^a$</td>
<td>41 ± 7$^a$</td>
<td>203 ± 25$^a$</td>
<td>91 ± 68$^a$</td>
<td>62 ± 12$^a$</td>
<td></td>
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<tr>
<td>AP (U/L)</td>
<td>104 ± 9</td>
<td>122 ± 11</td>
<td>168 ± 45</td>
<td>201 ± 44$^a$</td>
<td>299 ± 55$^a$</td>
<td>199 ± 38$^a$</td>
<td>232 ± 10$^a$</td>
<td>104 ± 7</td>
<td></td>
</tr>
<tr>
<td>Bile acids (µmol/L)</td>
<td>11 ± 3</td>
<td>87 ± 17$^a$</td>
<td>57 ± 17$^a$</td>
<td>367 ± 32$^a$</td>
<td>94 ± 21$^a$</td>
<td>310 ± 100$^a$</td>
<td>153 ± 40$^a$</td>
<td>94 ± 12$^a$</td>
<td></td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>2.1 ± 2$^a$</td>
<td>5 ± 0.2$^a$</td>
<td>5.6 ± 2.2$^a$</td>
<td>5 ± 2.5$^a$</td>
<td>0.9 ± 0.3$^a$</td>
<td></td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>38 ± 2</td>
<td>48 ± 2</td>
<td>43 ± 4</td>
<td>35 ± 5</td>
<td>103 ± 33$^a$</td>
<td>53 ± 8</td>
<td>40 ± 3</td>
<td>46 ± 2</td>
<td></td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.5 ± 0.05</td>
<td>0.4 ± 0.02</td>
<td>0.3 ± 0.02</td>
<td>0.6 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.03</td>
<td>0.3 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Well-established CCA was used for comparative purposes. Values are mean ± SEM from ≥5 animals per group. Abbreviations: ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; BDL, bile duct ligation; CCA, cholangiocarcinoma; GGT, gamma-glutamyl transferase.

$^aP < 0.05$ as compared with controls.
Glutathione S-transferase-pi (GSTpi), the predominant isoform in biliary epithelium of the phase II-detoxification GST enzymes, is overexpressed in cholangiocarcinoma (20), but is also elevated in animal models of hepatocellular carcinoma (15). The abundance of GSTpi mRNA (Fig. 2C) and protein (Fig. 2F) increased in thioacetamide groups. In thioacetamide-induced cholangiocarcinoma, we observed an overexpression of this enzyme, higher than that found in hepatocellular carcinoma (Fig. 2F), used here as a positive control. BDL alone or in combination with thioacetamide was not able to significantly upregulate GSTpi, although a trend toward a moderate increase in GSTpi mRNA was observed. On the other hand, the expression of GST\(\alpha\), the major GST isoform in the healthy liver parenchyma predominantly located in hepatocytes, was slightly reduced by thioacetamide administration, whereas a significant and progressive downregulation was observed in the BDL group (Fig. 2D and 2F). GST\(\alpha\) mRNA was also significantly reduced in the thioacetamide + BDL group. In cholangiocarcinoma, the abundance of GST\(\alpha\) mRNA was similar to that seen in healthy liver, but protein levels were markedly reduced (Fig. 2F).

\(\alpha\)-fetoprotein (AFP), a commonly used marker for hepatocellular carcinoma, was detected in fetal rat liver, used here as a positive control, but not in cholangiocarcinoma and samples obtained from any other group (Fig. 2F). These results demonstrated that, under the experimental conditions used here, thioacetamide induced the development of cholangiocarcinoma.

Figure 2. Determination of tumor markers. Expression of bile duct proliferation markers in sham-operated rats (Control) and animals with thioacetamide treatment (TAA) for 4 or 8 weeks, BDL 4 or 8 weeks before sample collection, or both (TAA + BDL) For comparison, cholangiocarcinoma (CCA) induced by thioacetamide treatment for 30 weeks is also included. CK-7 (A), claudin-4 (B), GSTpi (C), GST\(\alpha\) (D), and Neu (E) mRNA abundance was measured by RT-qPCR. Values are means ± SEM (n = 6 analyzed in triplicate). Results were normalized to \(\beta\)-actin mRNA. *, P < 0.05, compared with controls. F, representative Western blot analysis of liver homogenates from the same experimental groups. As positive controls, rat placenta for CK-7, LS174T cells for claudin-4, rat fetal liver for AFP, rat control liver for GST\(\alpha\), and rat hepatocellular carcinoma for GSTpi were used.
cholangiocarcinoma, but not hepatocellular carcinoma. This was confirmed by the immunolocalization of CK-7, claudin-4, and GSTpi (Fig. 3).

The increased expression or activation of the EGF receptor 2 (ErbB2 or Neu) has been associated with cell invasion, progression, and differentiation of cholangiocarcinoma (21, 22). Administration of thioacetamide increased Neu mRNA levels 2- and 5-fold after 4 and 8 weeks, respectively, although the change was only significant after 8 weeks. The levels of this protooncogene in thioacetamide-induced

![Figure 3. Tissue localization of markers. Immunofluorescence of CK-7, claudin-4, and GSTpi in rat liver cryosections of sham-operated rats (Control), or animals undergoing treatment with thioacetamide (0.05% in drinking water for 8 weeks) alone ([TAA], BDL 8 weeks before sample collection, or TAA + BDL. For comparison, an additional group with well-established intestinal-type cholangiocarcinoma (CCA) due to thioacetamide treatment for 30 weeks was included. Bar: 50 μm.)](image-url)
cholangiocarcinoma were increased more than 100-fold. BDL induced a marked overexpression of this gene. Combination of BDL with thioacetamide did not further enhance the stimulatory effect of BDL alone (Fig. 2E).

**Apoptosis, inflammation, and chemoprotection**

When Bax-α and Bcl-2 mRNA were measured (Fig. 4A and B), a similar induction of Bax-α and Bcl-2 was observed in animals receiving thioacetamide alone for 4 and 8 weeks, resulting in a non-significant change in the Bax-α/Bcl-2 ratio (Fig. 4C). BDL induced a marked overexpression of Bcl-2 after 4 weeks, whereas the abundance of Bax-α mRNA was similar to that seen in control livers, which resulted in a decrease in the Bax-α/Bcl-2 ratio (Fig. 4C). In the thioacetamide + BDL group, a 2.5-fold increase in Bax-α together with a 6-fold increase in Bcl-2 after 4 weeks were observed, whereas the results observed after 8 weeks were similar to those found in cholangiocarcinoma, i.e., no change in Bax-α mRNA but enhanced Bcl-2 levels, which resulted in a reduced Bax-α/Bcl-2 ratio (Fig. 4C), suggesting a prevalence of survival versus apoptosis. This was consistent with caspase-3 activity (Fig. 4D), which was significantly enhanced only in thioacetamide groups. Caspase-8 activity was not changed in any experimental group (data not shown).

Interleukin (IL)-6 is a multifunctional cytokine produced by a wide variety of cells that mediate inflammatory reactions. This cytokine has been found increased in the biliary tract and the systemic circulation of patients with cholangiocarcinoma (23). In vitro studies have revealed that IL-6 is able to stimulate cholangiolar tumor cells growth (24). In the present study, IL-6 expression in liver tissue samples collected from each experimental group was determined to assess whether the cholangiocarcinogenic effect of bile acid accumulation could be related with increased levels of this cytokine. Thioacetamide administration alone for 4 and 8 weeks did not induce changes in the expression of IL-6 (Fig. 5A). In contrast this was enhanced in all groups with BDL and also in cholangiocarcinoma (Fig. 5A).

To evaluate whether impaired FXR-mediated chemoprotection could be involved in enhanced susceptibility to cholangiocarcinoma development, the expression of this nuclear receptor was also determined. Thioacetamide had no or moderate inhibitory effect on FXR expression when administered alone for 4 or 8 weeks, respectively (Fig. 5B). In contrast, a dramatic decrease in FXR expression in all BDL groups and in cholangiocarcinoma was found (Fig. 5B).
Discussion

It is well known that livers affected by diseases characterized by prolonged cholestasis and inflammation are more prone to developing cholangiocarcinoma (1, 2), which suggests that bile acids could play a role in cholangiocarcinogenesis, as has also been suggested for other gastrointestinal tumors (3). Results of the present study indicate that bile acids are not primary genotoxic/carcinogenic agents but rather they are promoter/cocarcinogenic compounds involved in favoring cholangiocarcinoma development.

To elucidate whether direct carcinogenic effect of bile acids could be involved, we used a strategy based on bacterial SOS response to DNA damage (25), which consists in a cascade of events affecting a number of genes, with LexA playing a key role as a repressor for all genes of this system, and RecA encoding a protein that promotes the inactivation of the repressor when the SOS-inducing signal is triggered. We observed that none of the molecular species of bile acids assayed, nor thioacetamide, induced the SOS response in E. coli. In contrast, both cisplatin and thioacetamide metabolites were able to activate RecApr. Moreover, bile acids did not enhance or prevent the genotoxic effect of cisplatin. These results are consistent with previous studies reporting that sodium taurocholate and sodium deoxycholate are not able to trigger the bacterial SOS response by themselves (26). These results are consistent with previous studies reporting that sodium taurocholate and sodium deoxycholate are not able to trigger the bacterial SOS response by themselves (26). This may be due to the lack of a direct action of bile acids on DNA. Indeed, DNA–bile acid adducts have not been observed either after in vivo treatment of human cancer cell lines or in in vivo studies in rats (27).

Using bacterial tests, bile acids at very high concentrations (1–10 mmol/L) have been found to enhance the effect induced by fecal mutagens (26). Moreover, bile acid–induced DNA damage was found when colonocytes were incubated with DCA or CDCA. In another study, this was attributed not to a direct effect but rather to the generation of oxidative stress due to the high concentration of bile acids (600 μmol/L) used (28). Our results with several bile acid species at levels ranging from subtoxic (50 μmol/L) to moderately toxic (1,000 μmol/L) concentrations support the concept that a direct mutagenic effect of these compounds is unlikely.

About the in vivo experimental model used here to evaluate the promoter effect of bile acid accumulation in cholangiocarcinoma development, we found a marked interindividual variability in the carcinogenic response to thioacetamide, which may account for the discrepancies previously reported on the onset of cholangiocarcinoma in rodents using long-term administration of this carcinogenic agent (10–12, 29). Both cholangiocarcinoma and hepatocellular carcinoma have been reported to appear in the liver of rodents treated with thioacetamide (30, 31). Our results confirmed the ability of thioacetamide to induce cholangiocarcinoma but, at least in our experimental setting, no induction of hepatocellular carcinoma was observed.

Cholestasis alone induced massive bile duct proliferation together with upregulation of Neu and resistance to apoptosis. Although the combination of BDL with thioacetamide for 8 weeks also induced a ductal response, this was weaker than that induced by BDL alone. A reduction in BDL-induced bile duct proliferation has also been reported when combining cholestasis with other hepatotoxins such as carbon tetrachloride (32). The administration of thioacetamide alone during the same time, however, enhanced GSTpi expression and maintained both the apoptosis resistance and the overexpression of Neu. Interestingly, this has been observed in small hyperplastic biliary ducts in some cases of hepatolithiasis, and in most cases of primary sclerosing cholangitis (21). One possible explanation for this observation is that the toxic effect of thioacetamide could be partially overcome by the stimulation of cell proliferation induced by BDL.

Thioacetamide administration induced both apoptosis and survival signals, with a trend to increase the Bax-a/Bcl2 ratio, which may be interpreted in the sense that apoptosis prevailed over survival. However, in animals treated with thioacetamide for 30 weeks clear signs of overcoming apoptosis in tumor cells were observed, an event that was also seen much earlier when thioacetamide was combined with
Bile Acids in Cholangiocarcinogenesis

BDL. Thus, cholangiofibrosis, a lesion that generally progresses toward the formation of cholangiocarcinoma, was clearly observed only in thioacetamide + BDL combination. Thus, in the light of the classical three-stage model of chemically induced liver carcinogenesis our results indicate that bile acids may act as cell growth stimulating agents, which need the presence of a DNA-damaging initiator to promote cancer development. This promoter effect of bile acids may be due to the inhibition of apoptosis and/or to the activation of cell proliferation, which may increase the susceptibility to mutagens and the appearance of neoplastic transformation (33). Several mechanisms have been proposed to account for enhanced cell proliferation. These include activation of proto-oncogenes, such as c-fos and c-jun (34), and the stimulation of cyclooxygenase-2 activity (35), with the consequent increase in prostaglandin E2 production, which is involved in inducing the resistance of malignant cells to apoptosis and enhancing their invasiveness. Our results indicated that bile acid accumulation might favor cholangiocarcinoma development by stimulation of proinflammatory mechanisms and by impairing FXR-mediated chemoprotection against genotoxic insults. In conclusion, the accumulation of bile acids in liver tissue seems to have no direct carcinogenic effect but may favor cholangiocarcinoma development by playing a cocarcinogenic role due to stimulation of bile duct proliferation, enhanced inflammation and reduction in FXR-dependent chemoprotection.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Lozano, L. Sanchez-Vicente, M.J. Monte, E. Herraez, O. Britz, R.I.R. Macias
Study supervision: E. Lozano, L. Sanchez-Vicente, E. Herraez, O. Britz, J.M. Banales, J.J.G. Marin, R.I.R. Macias

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