Sulfasalazine reduces bile acid-induced apoptosis in human hepatoma cells and perfused rat livers

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Abbreviations: 5-ASA, 5-aminosalicylic acid; GCDCA, glycochenodeoxycholic acid; GPT, glutamate-pyruvate transaminase; LDH, lactate dehydrogenase; NF-κB, nuclear factor κB; ROS, reactive oxygen species; SPD, sulfapyridine; SSZ, sulfasalazine; STSP, staurosporine.

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ABSTRACT

Background: Bile acid-induced apoptosis in hepatocytes can be antagonized by NF-κB-dependent survival pathways. Sulfasalazine modulates NF-κB in different cell types. We aimed to determine the effects of sulfasalazine and its metabolites sulfapyridine and 5-aminosalicylic acid (5-ASA) on bile acid-induced apoptosis in hepatocytes.

Methods: Apoptosis was determined by caspase assays and immunoblotting, NF-κB activation by EMSA and reporter gene assays, generation of reactive oxygen species (ROS) fluorometrically, bile secretion gravimetrically and bile acid uptake radiochemically and by gaschromatography in HepG2-Ntcp cells and isolated perfused rat livers.

Results: Glycochenodeoxycholic acid (GCDCA, 75µmol/L)-induced apoptosis was reduced by sulfasalazine dose-dependently (1-1000 µmol/L) in HepG2-Ntcp cells, whereas its metabolites 5-ASA and sulfapyridine had no effect. Sulfasalazine significantly reduced GCDCA-induced activation of caspases 9 and 3. In addition, sulfasalazine activated NF-κB, and decreased GCDCA-induced generation of ROS. Bile acid uptake was competitively inhibited by sulfasalazine. In perfused rat livers, GCDCA (25 µmol/L)-induced liver injury and extensive hepatocyte apoptosis were significantly reduced by simultaneous administration of 100 µmol/L sulfasalazine: LDH and GPT activities were reduced by 82% and 87%, respectively, and apoptotic hepatocytes were observed only occasionally. GCDCA uptake was reduced by 45±5% when sulfasalazine was coadministered. However, when 50% of GCDCA (12.5 µmol/L) were administered alone, marked hepatocyte apoptosis and liver injury were again observed questioning the impact of reduced GCDCA uptake for the antiapoptotic effect of sulfasalazine.

Conclusion: Sulfasalazine is a potent inhibitor of GCDCA-induced hepatocyte apoptosis in vitro and in the intact liver.
INTRODUCTION

Cholestasis is a common feature of many human liver diseases. Elevated bile acid concentrations in hepatocytes, a hallmark of cholestasis, promote liver cell death resulting in liver injury and liver cirrhosis. Toxic bile acids induce hepatocellular apoptosis, thereby providing a cellular mechanism for bile acid–mediated liver injury. The glycine and taurine conjugates of chenodeoxycholic acid (GCDCA, TCDCA) are the predominant dihydroxy bile acids in cholestatic patients and have been held responsible for cholestasis-associated liver injury. GCDCA is thought to induce hepatocyte apoptosis by a Fas death receptor-dependent process, that is independent of Fas ligand, but induces oligomerization of Fas by increasing cell surface trafficking of Fas. GCDCA-induced generation of reactive oxygen species followed by epidermal growth factor receptor (EGF-R)-dependent tyrosine phosphorylation of Fas also appears to be required for GCDCA-induced Fas-signaling. The transcription factor NF-κB has been shown to reduce hepatocyte apoptosis induced by toxic bile acids, tumor necrosis factor-α (TNF-α) and during liver regeneration. Thus, NF-κB is an important factor of several anti-apoptotic signaling cascades in the liver.

Sulfasalazine was synthesized in 1942 to combine an antibiotic, sulfapyridine (SPD), and an antiinflammatory agent, 5-aminosalicyclic acid (5-ASA), for the treatment of rheumatoid arthritis. Later, sulfasalazine was also used successfully in the treatment of inflammatory bowel diseases. Although this drug has been used for decades, its mechanisms of action remain a matter of debate. Numerous pharmacological and biochemical effects have been described, including modulatory effects on leukocyte function. Recently, it has been shown that sulfasalazine is a potent and specific inhibitor of NF-κB in human colon epithelial cells. In these cells, sulfasalazine seems to be a direct inhibitor of IκB kinases α and β by antagonizing adenosine triphosphate binding. However, it is not known if sulfasalazine or its metabolites can also modify NF-κB signaling in hepatocytes.

The overall objectives of this study were, therefore, to examine the effects and potential mechanisms of sulfasalazine and its metabolites on GCDCA-induced apoptosis in hepatocytes. To address these objectives we used a human hepatoma cell line stably transfected with the bile acid transporter sodium taurocholate cotransporting polypeptide (Ntcp) and isolated perfused rat livers.

MATERIAL AND METHODS

Reagents – ZVAD-FMK was from Promega (Madison, WI). 5-(and-6)-carboxy-2’7’-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) was from Molecular Probes (Eugene, OR). [³H]-Taurocholate was from Perkin Elmer (Boston, MA). Antibodies against cleaved caspase 9 and cleaved caspase 3 were purchased from Cell Signaling (Beverly, MA). MG132 was from Calbiochem (La Jolla, CA). GCDCA, sulfasalazine, sulfapyridine, 5-aminosalicyclic acid, dimethyl sulfoxide (DMSO), staurosporine and all other reagents were obtained from Sigma Chemical (St. Louis, MO).

Cell Culture - HepG2-Ntcp cells were grown at 37°C under 5% CO₂ in MEM (pH 7.4) containing 10% foetal bovine serum, 1% nonessential amino acids, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B.

[³H]Taurocholic acid uptake - Confluent HepG2-Ntcp cells were washed with a buffer containing 100 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5.5 mmol/L d-
glucose and 10 mmol/L N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (pH 7.5; 37°C). After incubation in NaCl medium containing 1 µCi/mL [3H]taurocholic acid (TCA) and 10 µmol/L unlabelled TCA at 37°C for 20 min, cells were washed with an ice-cold NaCl medium containing 1 mmol/L unlabelled TCA and lysed with 0.5 mL Triton X-100 (1%, v/v). Aliquots of 400 µL were dissolved in 10 mL scintillation cocktail (Ultima Gold Canberra Packard, Frankfurt/Main, Germany). Radioactivity was quantified using a liquid scintillation analyser (Packard Instrument Co., Frankfurt, Germany).

**Caspase assays** – Caspase 3/7, and caspase 9 activation were determined in subconfluent HepG2-Ntcp cells treated with GCDCA in the absence or presence of sulfasalazine or the pancaspase inhibitor ZVAD-FMK at the indicated concentrations and time intervals. Commercially available caspase assay kits from Promega (Madison, WI) were performed according to the recommendations of the manufacturer.

**Plasmids and Transfection** – Luciferase reporter plasmids p105 (cona-luc) and p106 (κB-cona-luc) for NF-κB reporter gene assays have been previously described. The TK-Renilla-CMV plasmid was purchased from Promega and used to normalize for transfection efficiency in luciferase assays. HepG2-Ntcp cells at a confluence of approximately 50% were transiently transfected using FuGENE (Roche, Mannheim, Germany) and used 48 h after transfection.

**Electrophoretic Mobility Shift Assay (EMSA)** – HepG2-Ntcp cells were stimulated with diluent or sulfasalazine at different concentrations. Six µg of nuclear proteins and 3 µg of the nonspecific competitor poly(dIdC) were incubated in binding buffer (100 mM HEPES, 300 mM KCl, 20% Ficoll, 0.05% NP-40, 0.5 mg/ml BSA) with 3.5 pmol of double-stranded DNA oligonucleotide containing a NF-κB-consensus binding sequence (5'-AGT TGA GGG GAC TTT CCC AGG C-3') that was labeled with [γ-32P]-ATP using T4 polynucleotide kinase (Promega). Protein-DNA complexes were separated from the unbound DNA probe by electrophoresis through 5% native polyacrylamide gels.

**Luciferase Reporter Gene Assay** – HepG2-Ntcp cells were cotransfected with 0.2 µg of TK-Renilla-CMV and 1.5 µg of either p105 or p106. Forty-eight hours later, the cells were cultured in serum-free MEM for 18-24 h and then stimulated with bile acids and/or sulfasalazine for 1 h. Both firefly and Renilla luciferase activities were quantitated using dual reporter gene assays from Promega according to the manufacturer’s instructions using a TD 20/20-Luminometer (Software Turner Design Version 2.0.1, Turner Designs Inc., CA). Background luciferase expression, as determined in cells transfected with p105, was subtracted from p106 values.

**Measurement of reactive oxygen species (ROS)** – Confluent HepG2-Ntcp cells were incubated with carboxy-H₂DCFDA (4 µmol/L) for 4 h at 37°C, washed three times, incubated with GCDCA in the absence or presence of sulfasalazine at the indicated concentrations for 2 h at 37°C, and quantitated in a CytoFluor 4000 reader (Perseptive Biosystems, Weiterstadt, Germany) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

**Immunoblot analysis** – Subconfluent cells were treated with staurosporine (5 µmol/L) or GCDCA (75µmol/L) in the absence or presence of sulfasalazine, washed with PBS, homogenized in ice-cold lysis buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 2 mM EDTA, 1% Triton, 100 µM Vanadate, 10 mM NaF), incubated for 5 min on ice, sonicated and centrifuged for 5 min at 14000 g and 4°C. The supernatant was resolved by 12.5% SDS-PAGE, transferred to Immobilon-P membranes (Millipore, Eschborn, Germany) and probed against the appropriate primary antibody at a dilution of 1:1000 in 5% milk/TBS-T overnight. Peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) was incubated at a dilution of 1:4000. Membranes were stripped and reprobed with anti-β-actin antibody (1:3500, Sigma) to ensure equal loading.
Animals - Male Sprague-Dawley rats (229±16 g) were obtained from Charles River (Sulzfeld, Germany). They were subjected to a 12-h day-night rhythm with unlimited access to food and water.

Isolated Rat Liver Perfusion - The technical procedure used has been described previously. In brief, livers were perfused in a non-recirculating fashion with Krebs-Ringer bicarbonate solution at 37°C at a constant flow rate of 4.0-4.5 ml/min/g liver for 90 min. After 20 min, sulfasalazine (or the carrier DMSO only, 0.001%, v/v) was continuously infused for 70 min to reach a final concentration of 100 µmol/L in the portal vein. After 30 min, the bile acid GCDCA (or the carrier DMSO only, 0.1%, v/v) was infused for 60 min at a continuous rate to reach a final concentration of 25 µmol/L or 12.5 µmol/L in the portal vein. Hepatovenous efflux of LDH and GPT as indicators of liver cell damage were measured by use of standard enzymatic tests. Bile flow was measured gravimetrically in prepared tubes.

Immunofluorescence microscopy - Activated caspase 3 and cytokeratin intermediate filament (CK-IF) alterations typical for apoptotic cell death were studied as described. For quantification, caspase 3 positive hepatocytes with concomitant CK-IF breakdown were counted in 20 different high-power fields per sample and expressed as x-fold increase over control.

Determination of bile acids – GCDCA concentrations in the hepatovenous effluents were determined as described previously. Briefly, bile acids were extracted with Bond-Elut C18 cartridges (Analytichem International, San Diego, CA). Deconjugated bile acids were isolated by extraction on Lipidex 1000 (Packard Instruments, Groningen, The Netherlands) and were then methylated and trimethylsilylated. Capillary gas chromatography was performed using a Carlo Erba Fractovap 4160 analyser (Carlo Erba, Hofheim, Germany). Bile acid derivatives were separated on a silica capillary CP Sil 19 CB column (Chrompack, Middelburg, The Netherlands). Eluting bile acid derivatives were detected by a flame ionization detector.

Statistics – Results from at least 3 independent experiments are expressed as means ± SD. Differences between groups were compared using an analysis of variance for repeated measures (ANOVA) and a post hoc Bonferroni test to compare for multiple comparisons.

RESULTS

Do sulfasalazine or its metabolites modulate bile acid-induced apoptosis in vitro? – The bile acid transporting human hepatoma cell line HepG2-Ntcp was used for the in vitro studies. GCDCA effectively induced apoptosis in this cell line while parent non-bile acid transporting HepG2 cells were not sensitive to GCDCA-induced apoptosis (Fig. 1A). Based on these results, 75 µmol/L GCDCA was used for the remainder of the in vitro studies.

A dose-dependent reduction of GCDCA-induced apoptosis was observed when sulfasalazine was used in combination with 75 µmol/L GCDCA. After 4 hours, sulfasalazine at 1000 µmol/L reduced GCDCA-induced caspase 3/7-activity to control levels, 100 µmol/L resulted in a 80±12% reduction and 10 µmol/L in a 25±7% reduction of caspase 3/7 activity (Fig. 1B). Sulfasalazine at 1 µmol/L had no effect on GCDCA-induced apoptosis. Because HepG2-Ntcp is a hepatoma cell line, we also repeated this experiment using 18-hour cultured primary mouse hepatocytes and confirmed the dose dependent antiapoptotic effect of sulfasalazine on GCDCA-induced apoptosis which was nearly identical to the one shown in Fig. 1B (n=3; data not shown).

Hepatocytes are considered to be type II cells in which the mitochondrial pathway is essential to induce apoptosis. Therefore, the effects of sulfasalazine on GCDCA-induced caspase 9 activation were examined. In these experiments, sulfasalazine reduced GCDCA-
induced caspase 9 activation in a dose-dependent manner similar to the reduction of the effector caspases 3/7 shown above (Fig. 1C).

We next evaluated the effects of the two metabolites of sulfasalazine, 5-aminosalicylic acid (5-ASA) and sulfapyridine (SPD), in the same experimental system. In contrast to sulfasalazine, neither 5-ASA nor SPD had any effect on GCDCA-induced apoptosis as measured by caspase 3/7 activity (Fig. 2 A and B). The combination of 5-ASA and SPD in the same ratio as present in sulfasalazine did also not alter GCDCA-induced apoptosis (data not shown). These data indicate that sulfasalazine, but not its metabolites, is a potent inhibitor of GCDCA-induced apoptosis in hepatocytes.

Is NF-κB activity in HepG2-Ntcp cells induced by sulfasalazine? – The effect of sulfasalazine on NF-κB activation in HepG2-Ntcp cells was examined by EMSA and luciferase reporter gene assays. Sulfasalazine induced NF-κB activity in a dose dependent manner in HepG2-Ntcp cells after 1 h of incubation (Fig. 3A). NF-κB activation by 100 and 1000 µmol/L sulfasalazine could also be shown by EMSA (Fig. 3A, inset). In comparison, GCDCA at 75 µmol/L did not significantly affect NF-κB activity. Thus, sulfasalazine appears to be an inducer of NF-κB in hepatoma cells. To assess the potential role of sulfasalazine-induced NF-κB activation for its beneficial effect on GCDCA-induced apoptosis, the NF-κB inhibitor MG132 (carbobenzoxy-leucinyl-leucynil-leucynal)\textsuperscript{20} was used for additional experiments. At a concentration of 50 µmol/L, MG132 reduced sulfasalazine-induced NF-κB activation to control levels as demonstrated by EMSA (Fig. 3B) and reporter gene assays (not shown). However, the effect of sulfasalazine on GCDCA-induced caspase 3/7-activity was not altered by MG132 (Fig. 3C), indicating that this mechanism is not relevant for the beneficial effects of sulfasalazine on GCDCA-induced apoptosis.

Does sulfasalazine reduce GCDCA-induced ROS generation? – To determine a possible effect of sulfasalazine on GCDCA-induced ROS-generation, HepG2-Ntcp cells were loaded with the fluorescent dye carboxy-H\textsubscript{2}-DCFDA and then incubated with GCDCA (75 µmol/L) in the absence or presence of sulfasalazine. GCDCA significantly increased generation of ROS by nearly 30% compared to controls after 2 hours. Combination of GCDCA with sulfasalazine (1-1000 µmol/L) led to a dose-dependent reduction of ROS generation, which reached control levels using sulfalazine at 1000 µmol/L (Table 1). Since the pro-apoptotic Bcl-2 proteins Bax and Bid have also been shown to be important for the mitochondrial damage during bile acid-induced apoptosis\textsuperscript{21}, additional immunoblot experiments were performed. However, expression and activation of both, Bax and Bid, were not modulated by sulfasalazine (data not shown).
Table 1. Sulfasalazine reduces GCDCA-induced oxidative stress in HepG2-Ntcp cells.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Fold ROS formation</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td>GCDCA 75 µmol/L</td>
<td>1.28 ± 0.05 a</td>
</tr>
<tr>
<td>Sulfasalazine 1000 µmol/L</td>
<td>0.97 ± 0.02</td>
</tr>
<tr>
<td>GCDCA 75 µmol/L + Sulfasalazine 1 µmol/L</td>
<td>1.28 ± 0.04</td>
</tr>
<tr>
<td>GCDCA 75 µmol/L + Sulfasalazine 10 µmol/L</td>
<td>1.24 ± 0.01</td>
</tr>
<tr>
<td>GCDCA 75 µmol/L + Sulfasalazine 100 µmol/L</td>
<td>1.09 ± 0.03 b</td>
</tr>
<tr>
<td>GCDCA 75 µmol/L + Sulfasalazine 1000 µmol/L</td>
<td>1.00 ± 0.03 c</td>
</tr>
</tbody>
</table>

NOTE: HepG2-Ntcp cells were loaded for 4 h with the fluorescent dye carboxy-H$_2$-DCFDA (4 µmol/L) to detect generation of reactive oxygen species (ROS). Then, cells were incubated with GCDCA in the absence or presence of sulfasalazine for 2 h. Data represent the n-fold increase in carboxy-H$_2$-DCFDA fluorescence compared to control (set as 1). Results are the mean ± S.D. of 12 independent experiments. a, p< 0.01 vs. control; b, p<0.05 vs. GCDCA; c, p<0.01 vs. GCDCA.

Is bile acid uptake inhibited by sulfasalazine? – We next evaluated if sulfasalazine influences bile acid uptake as another possible mechanism for its anti-apoptotic properties. Bile acid uptake under control conditions was set as 100%. Sulfasalazine at a concentration of 1 µmol/L had no effect and at 10 µmol/L tended to reduce bile acid uptake in HepG2-Ntcp cells, but the difference did not reach significance (Fig. 4A). However, using 100 µmol/L sulfasalazine, bile acid uptake was reduced to 71±12 % compared to control and to 23±7% at 1000 µmol/L, both significant differences. In contrast, 5-ASA as well as sulfapyridine did not affect bile acid uptake (Fig. 4A).

To determine the mechanism of sulfasalazine-induced reduction of bile acid uptake, kinetics of bile acid uptake in the absence or presence of sulfasalazine were evaluated. The Km under control conditions was 42 µmol/L and increased to 99 µmol/L (at 10 µmol/L SSZ) and to 263 µmol/L (at 100 µmol/L SSZ). Vmax values were nearly unchanged (0.24±0.03 nmol/min/mg protein) under all experimental conditions. Inhibition was thus competitive as also shown in the Lineweaver-Burk graph (Fig. 4B).
**Does sulfasalazine also inhibit apoptosis that is not induced by bile acids?** – Stauorosporine, a nonspecific kinase inhibitor, induces apoptosis through the disruption of mitochondrial function. At a concentration of 5 µmol/L, stauorosporine readily induced cleavage of caspase 9 and caspase 3 in HepG2-Ntcp cells, which could be inhibited by the pancaspase inhibitor ZVAD-FMK (Fig. 5A). Treatment with stauorosporine and sulfasalazine (1-1000 µmol/L) resulted in a dose-dependent reduction of both caspase 9 and caspase 3 cleavage. Densitometric analysis of the cleaved caspase 3 immunoblots (normalized to β-actin) revealed that stauorosporine-induced caspase 3 cleavage was significantly reduced when 100 or 1000 µmol/L sulfasalazine was administered (Fig. 5B). Similar results were obtained for cleaved caspase 9 (data not shown). Sulfasalazine also dose-dependently reduced caspase 3/7-activity, when TNF-α and actinomycin D were used as an apoptotic stimulus that requires Fas-signaling (Fig. 5C). These results indicate that inhibition of intracellular apoptosis signaling might be more important for the antiapoptotic effects of sulfasalazine than the observed reduction of bile acid uptake.

**Does sulfasalazine ameliorate GCDCA-induced liver damage in the intact organ?** – Livers of Sprague-Dawley rats were perfused with sulfasalazine (100 µmol/L) or the carrier DMSO only (0.1%, v/v) for 70 minutes beginning at minute 20. GCDCA (25 µmol/L) was infused for 60 minutes beginning at minute 30. We have recently demonstrated that GCDCA (25 µmol/L) induces widespread hepatocyte apoptosis and liver damage in perfused rat livers. In contrast, GCDCA-induced apoptosis and liver injury were significantly reduced by simultaneous perfusion with 100 µmol/L sulfasalazine: LDH and GPT activities were reduced by 82% and 87%, respectively, as compared to GCDCA-perfused livers (Fig. 6A). Correspondingly, apoptotic hepatocytes were only observed occasionally compared to the extensive hepatocyte apoptosis (>100 fold compared to control) observed after treatment with GCDCA alone (Fig. 6B). Sulfasalazine by itself did not cause liver damage or hepatocyte apoptosis. Thus, sulfasalazine distinctly reduces GCDCA-induced apoptosis and liver damage also in the intact liver.

**Is GCDCA-induced cholestasis reversed by sulfasalazine?** – Bile flow was 1.31 ± 0.31 µl/min/g of liver (n=6) after 20 minutes before bile acids or their carrier DMSO (0.1% v/v) were infused, indicating an adequate secretory capacity of livers. GCDCA (25 µmol/L) reduced bile flow to 17% of controls (p<0.01 vs. control). Sulfasalazine alone (100 µmol/L) also reduced bile flow to 62% of controls (p<0.05 vs. control). In contrast, sulfasalazine markedly increased bile flow in GCDCA-treated livers to 79% of controls (p<0.05 vs. GCDCA; Fig. 7). Thus, sulfasalazine partially reverses GCDCA-induced cholestasis in the intact liver.

**Does sulfasalazine reduce bile acid uptake in the intact organ?** – We investigated the possible role of sulfasalazine-induced reduction of bile acid uptake as a major mechanism for its protective effect on GCDCA-induced liver damage in the perfused liver. Bile acid uptake was calculated by subtracting GCDCA concentration in the hepatovenous effluate from the concentration in the portal vein, because this difference likely reflects uptake of bile acids into the liver. Similar to the results observed in HepG2-Ntcp cells, GCDCA uptake was reduced by 45±5% after 45 min of perfusion with sulfasalazine (n=6) suggesting inhibition of bile acid uptake as a possible mechanism for the protective effect of sulfasalazine (Fig. 8A). Based on this experiment we next evaluated if half the dose of GCDCA (12.5 µmol/L) – which is presumably taken up by the liver during concomitant administration of sulfasalazine and 25 µmol/L GCDCA – still causes liver damage and hepatocyte apoptosis. If inhibition of uptake would be the major
mechanism of protection, liver damage should not occur using 12.5 µmol/L GCDCA. However, when 12.5 µmol/L GCDCA was used, marked hepatocyte apoptosis (Fig. 8B) and liver injury (GPT 40.7±9.5 mU/min/g) still occurred (Fig. 8C). Combination of 12.5 µmol/L GCDCA and 100 µmol/L sulfasalazine again significantly reduced liver damage and hepatocyte apoptosis (data not shown). Thus, inhibition of bile acid uptake appears to play only a minor role in the protective effects of sulfasalazine in the intact liver.

DISCUSSION

The principle findings of this study indicate that sulfasalazine is a potent inhibitor of bile acid-mediated hepatocyte apoptosis in vitro and in the intact liver. In addition, sulfasalazine ameliorates GCDCA-induced cholestasis in perfused rat livers. These results suggest that sulfasalazine may be of potential benefit in the treatment of cholestatic liver diseases by reducing liver injury and cholestasis.

At present, there is no data concerning the effects of sulfasalazine on apoptosis in hepatocytes. In T-lymphocytes, sulfasalazine has been shown to induce apoptosis by caspase-independent mechanisms. In a recent study, sulfasalazine promoted hepatic stellate cell apoptosis in vitro and in vivo. In human glioma cells and colon carcinoma cell lines, sulfasalazine inhibited Fas-mediated apoptosis but simultaneously sensitized the cells to TRAIL-mediated apoptosis. However, apoptosis could not be induced by sulfasalazine in SW620 colon carcinoma cells or primary human synoviocytes. There appears to be a cell type-specific sensitivity to sulfasalazine. In the present study, sulfasalazine inhibited Fas-dependent apoptosis, because GCDCA- as well as TNF-α/actinomycin D-induced apoptosis are considered Fas-dependent. Sulfasalazine also inhibited staurosporine-induced apoptosis, suggesting that its protective effect is not limited to a reduced formation of the death inducing signaling complex.

In contrast to the studies in colon epithelial cells, hepatic stellate cells and T-lymphocytes, sulfasalazine did induce NF-κB activity in our model. However, this NF-κB activation appears not to be responsible for the observed beneficial effects of sulfasalazine, because GCDCA-induced apoptosis was unchanged when sulfasalazine was administered together with the NF-κB inhibitor MG132. Interestingly, sulfasalazine modulated apoptosis in a NF-κB-independent manner in human glioma cells, although sulfasalazine also significantly altered NF-κB activity in this model. However, sulfasalazine appears to have a protective effect on mitochondria, as suggested by its capacity to reduce GCDCA-induced ROS generation and caspase 9 activation, and by its reduction of staurosporine-induced cytotoxicity, a model of cell death known to be mediated by mitochondrial dysfunction. Thus, inhibition of the mitochondrial pathway might be partly responsible for the protective effect of sulfasalazine in our model. Interestingly, sulfasalazine has been described as a ROS scavenger in the past.

Sulfasalazine reduced bile acid uptake in hepatocytes at higher concentrations. This reduction is due to a competitive inhibition of the transfected bile acid transporter Ntcp. Since uptake of GCDCA is necessary to induce apoptosis, it could be speculated that this is the major mechanism of action in our model. However, at a concentration of 100 µmol/L sulfasalazine, 70% of the bile acids were still taken up by HepG2-Ntcp cells and 50 µmol/L GCDCA (equivalent to 75 µmol/L GCDCA in combination with 100 µmol/L sulfasalazine) readily induces hepatocyte apoptosis. In addition, sulfasalazine also inhibited staurosporine-induced apoptosis in HepG2-Ntcp cells. Thus, a direct effect on apoptosis signaling appears more important than the reduction in bile acid uptake, because staurosporine does not require active transport into cells. This conclusion is further supported by the perfusion studies. Sulfasalazine inhibited GCDCA-induced apoptosis almost to control levels, although bile acid uptake was only
reduced by approximately 50%. In addition, when GCDCA was used at half the concentration (12.5 versus 25 µmol/L), which is presumably still taken up by the hepatocytes during sulfasalazine administration, significantly more liver damage and hepatocyte apoptosis occurred compared to the combination of sulfasalazine with GCDCA 25 µmol/L. This strongly suggests that the major mechanism of action of sulfasalazine on reduction of GCDCA-induced liver damage is inhibition of apoptosis signaling. Reduction of bile acid uptake appears to be only a minor contributing mechanism.

Sulfasalazine partly reversed GCDCA-induced impairment of bile flow in our model. How can this observation be explained? GCDCA is a toxic bile acid that significantly decreases bile flow in part due to hepatocyte injury. Sulfasalazine protects the liver against GCDCA-induced injury, thereby restoring the capacity of the liver to generate bile. In addition, GCDCA might contribute to the bile acid-dependent bile flow when its cytotoxic effects are inhibited by sulfasalazine.

The two metabolites of sulfasalazine, 5-ASA and sulfapyridine, had no effect on GCDCA-induced apoptosis. Similar observations have also been made in other models. In colon epithelial cells, sulfasalazine but not its metabolites could suppress NF-κB activity and likewise, in T-lymphocytes only sulfasalazine induced apoptosis. Thus, only the intact molecule seems to modulate intracellular signaling. This observation appears to be of clinical significance, because only sulfasalazine, but not 5-ASA has beneficial effects in rheumatoid arthritis as a disease modifying agent.

The protective effects of sulfasalazine observed in the present study were most pronounced at concentrations of 100 and 1000 µmol/L. Early studies have shown that peak serum levels of approximately 100 µmol/L sulfasalazine are reached in serum of patients after oral administration. The concentration of sulfasalazine in the portal vein, which is crucial for the concentration reached in hepatocytes, is presumably even higher, because sulfasalazine undergoes enterohepatic circulation. Thus, our results may have therapeutic implications for patients with chronic cholestasis.

In summary, the data presented in the current study suggest that bile acid-mediated apoptosis can be effectively inhibited by sulfasalazine at concentrations that are reached during therapeutic application of this drug in patients. Together with the recent findings that sulfasalazine reduces hepatic fibrosis by promoting hepatic stellate cell apoptosis, our results have potential implications for reducing liver injury during cholestasis. This novel therapeutic strategy deserves further study, for example in patients with primary sclerosing cholangitis and ulcerative colitis which are concurrently treated with sulfasalazine.

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Competing interests: The authors have no competing interests to report.

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REFERENCES

FIGURE LEGENDS

Figure 1 – Sulfasalazine reduces bile acid-induced apoptosis in a hepatoma cell line. (A) HepG2-Ntcp and parent HepG2 cells were treated with diluent or GCDCA at the indicated concentrations for 4 hrs. The pancaspase inhibitor ZVAD-FMK (20 µmol/L) was used to demonstrate specificity of the assay. Apoptosis was quantified by measuring caspase 3/7 activity and expressed as percentage over control (set as 100%). (B) HepG2-Ntcp cells were treated with GCDCA (75 µmol/L) in the presence or absence of sulfasalazine at the indicated concentrations. Apoptosis was quantified by measuring caspase 3/7 activity (*, p< 0.05; #, p<0.01 vs. GCDCA). (C) HepG2-Ntcp cells were treated with GCDCA (75 µmol/L) in the presence or absence of sulfasalazine at the indicated concentrations. Apoptosis was quantified by measuring caspase 9 activity (*, p< 0.05; #, p<0.01 vs. GCDCA). Results are the mean ± S.D. of six independent experiments.

Figure 2 – 5-Aminosalicylic acid and sulfapyridine do not affect bile acid-induced apoptosis. HepG2-Ntcp cells were treated with diluent or GCDCA (75 µmol/L) in the presence or absence of 5-aminosalicylic acid (5-ASA) (A) or sulfapyridine (SPD) (B) at the indicated concentrations for 4 hrs. Apoptosis was quantified by measuring caspase 3/7 activity and expressed percentage over control (set as 100%). Results are the mean ± S.D. of three independent experiments.

Figure 3 – Sulfasalazine induces NF-κB activity. (A) Hep-G2-Ntcp cells were cotransfected with TK-Renilla-CMV and Luc-NF-κB (p106). Cells were stimulated with diluent (DMEM), sulfasalazine at the indicated concentrations or 75 µmol/L GCDCA for 1 h. Cell lysates were prepared, and firefly and Renilla luciferase assays were performed. To control for transfection efficiency, the ratio of firefly to Renilla luciferase was calculated. The resulting values are presented as arbitrary units. The inset shows an EMSA for NF-κB after stimulation of HepG2-Ntcp cells with 100 or 1000 µmol/L sulfasalazine or medium only (control) for 1 h. Results are the mean ± S.D. of three independent experiments (*, p<0.05; **, p<0.01; ***, p<0.001 vs. control). (B) HepG2-Ntcp cells were preincubated for 1 h with MG132 at the indicated concentrations and then SSZ (1000 µmol/L) or diluent (as control) were added for 1 h. Nuclear extracts were prepared, and EMSAs were performed. (C) HepG2-Ntcp cells were incubated with GCDCA, sulfasalazine or diluent (as control) in the absence or presence of the NF-κB inhibitor MG132 (50 µmol/L) for 4 hrs. Apoptosis was quantified by measuring caspase 3/7 activity and expressed as percentage over control (set as 100%). Results are the mean ± S.D. of three independent experiments.

Figure 4 – Sulfasalazine competitively inhibits bile acid uptake. (A) HepG2-Ntcp cells were incubated with [3H]-Taurocholic acid (1 mCi/ml) in the absence or presence of sulfasalazine (SSZ), 5-aminosalicylic acid (5-ASA) or sulfapyridine (SPD) at the indicated concentrations for 10 min. Cells were then washed, lysed and radioactivity measured in an automated counter. Results are the mean ± S.D. of six independent experiments and are expressed as percent of control (*, p<0.05; **, p<0.01 vs. control). (B) HepG2-Ntcp cells were incubated with [3H]-Taurocholic acid (1 mCi/ml) at different concentrations (0-100 µmol/L) in the absence or presence of sulfasalazine (10 and 100 µmol/L). The corresponding Km and Vmax values were calculated after measuring intracellular radioactivity and plotted as a Lineweaver-Burk graph.
Figure 5 - Sulfasalazine reduces staurosporine- and TNF-α/actinomycin D-induced apoptosis. (A) Subconfluent HepG2-Ntcp cells were treated with diluent or staurosporine (STSP) in the absence or presence of sulfasalazine (SSZ) or the pancaspase inhibitor ZVAD-FMK at the indicated concentrations for 4 hrs. Equivalent amounts of proteins were immunoblotted with antibodies against cleaved caspase 3 or cleaved caspase 9. Membranes were then stripped and reprobed with an anti-β-actin antibody to ensure equal loading in an identical procedure. Representative blots from 3 independent experiments are shown. (B) In addition, densitometry of cleaved caspase 3 and β-actin was performed and expressed as the ratio cleaved caspase 3/β-actin. Results are the mean ± S.D. of three independent experiments (*, p< 0.05; #, p<0.01 vs. STSP). (C) HepG2-Ntcp cells were treated with TNF-α (28 ng/mL) in combination with actinomycin D (0.2 µg/mL) in the presence or absence of sulfasalazine at the indicated concentrations. Apoptosis was quantified by measuring caspase 3/7 activity. Results are the mean ± S.D. of three independent experiments (*, p< 0.05 vs. GCDCA).

Figure 6 – Sulfasalazine reduces bile acid-induced liver injury and apoptosis in perfused rat livers. Rat livers were perfused with 25 µmol/L of GCDCA or the carrier DMSO only in the absence or presence of 100 µmol/L sulfasalazine for 60 minutes. (A) After 55 minutes of bile acid administration, LDH and GPT activities in the hepatovenous effluvate were determined photometrically. Results are the mean ± S.D. of 6 independent experiments. (B) Hepatocyte apoptosis was determined by immunohistochemistry of activated caspase 3 and double-immunofluorescence labeling for activated caspase-3 and cytokeratin (CK) 18. After 60 minutes, double-immunofluorescence labeling for CK 18 (green) and activated caspase 3 (red and yellow due to colocalization with CK 18) in GCDCA-treated livers reveals massive hepatocellular activation of caspase 3 in cells with CK intermediate filament breakdown and granular cytoplasmic condensation. In contrast, immunoreaction for activated caspase 3 is similar to controls in livers perfused with GCDCA and sulfasalazine (magnification x 400). Representative pictures from 6 independent experiments are shown.

Figure 7 – Sulfasalazine partially reverses GCDCA-induced cholestasis. DMSO (0.1% v/v), GCDCA, sulfasalazine (SSZ) and GCDCA in combination with SSZ were administered for 60 minutes (GCDCA) and 70 minutes (SSZ). GCDCA significantly reduced bile flow (p<0.01 vs. control). While SSZ alone decreased bile flow, SSZ significantly increased bile flow in GCDCA-treated livers (*, p<0.05 vs. control; **, p<0.01 vs. control; #, p<0.05 vs. GCDCA). Results are expressed as the mean ± S.D. of 6 experiments.

Figure 8 – Reduced bile acid uptake is not sufficient to explain the effects of sulfasalazine in the intact liver. Rat livers were perfused with GCDCA (12.5 or 25 µmol/L) or the carrier DMSO only in the absence or presence of 100 µmol/L sulfasalazine for 60 minutes. (A) After 15 minutes of bile acid administration, chenodeoxycholic acid concentrations were determined in the hepatovenous effluvate. The difference between portal vein concentration (concentration in the perfusate) and hepatic vein concentration (concentration in the effluvate) as an indirect measure of bile acid uptake into the liver is mapped on the y-axis. GCDCA at both concentrations was almost completely taken up into the liver, because it could only be detected in traces in the hepatovenous effluvate. In contrast, 11±1 µmol/L of chenodeoxycholic acid were detected in the hepatovenous effluvate when GCDCA (25 µmol/L) and sulfasalazine (100 µmol/L) were administered, indicating that nearly 50% of GCDCA were not taken up. Bile acid uptake into the liver was almost identical when GCDCA was administered at 12.5 µmol/L or was coadministered
with sulfasalazine (100 µmol/L) at 25 µmol/L. (B) Hepatocyte apoptosis was determined by immunohistochemistry of activated caspase 3 and cytokeratin (CK) 18 after 60 minutes of bile acid administration (magnification x 400). Hepatocyte apoptosis is markedly more pronounced with GCDCA 12.5 µmol/L compared to GCDCA 25 µmol/L in combination with 100 µmol/L sulfasalazine, although bile acid uptake into the liver is similar in both conditions. Representative pictures from 6 independent experiments are shown. (C) After 55 minutes of bile acid administration, GPT activities in the hepatovenous effluate were determined photometrically. Comparable to (B), GPT efflux as a marker of liver damage is significantly increased with GCDCA 12.5 µmol/L compared to GCDCA 25 µmol/L in combination with 100 µmol/L sulfasalazine (*, p<0.01). Results in panels A and C are the mean ± S.D. from 6 independent experiments.
Figure 2

A

GCDCA [μmol/L] - 75 75 75 75 75 75 75
5-ASA [μmol/L] - 1000 1 10 100 1000

B

GCDCA [μmol/L] - 75 75 75 75 75 75 75
SPD [μmol/L] - 1000 1 10 100 1000
Figure 3

A

![Bar graph showing NF-κB activity](#)

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B

![Western blot showing NF-κB expression](#)

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Figure 5

A

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B

![Bar graph showing cleaved Caspase 3/β-actin expression](image)

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Figure 5

C

Caspase 3/7 activity [%]

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4h
Figure 7

The figure shows a bar graph comparing bile flow [µL/50 min/g liver] among different groups:

- **Control**
- **SSZ 100 µmol/L**
- **GCDCA 25 µmol/L**
- **GCDCA 25 µmol/L + SSZ 100 µmol/L**

Significance levels indicated by asterisks:

- * p < 0.05
- ** p < 0.01
- # p < 0.05 compared to control