JPET Fast Forward. Published on November 14, 2003 as DOI:10.1124/jpet.103.060129
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The Caspase Inhibitor IDN-6556 Attenuates Hepatic Injury and Fibrosis in the Bile Duct Ligated Mouse [1]

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[1]This work was supported by a fellowship grant from the Postdoctoral Program of the German Academic Exchange Service to AC, NIH grant (DK063947) to GJG, and the Mayo Foundation.

JPET Fast Forward. Published on November 14, 2003 as DOI: 10.1124/jpet.103.060129
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Running title: Apoptosis and fibrosis

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Abbreviations used: ALT, alanine aminotransferase; BDL, bile duct ligation/ligated; HSC, hepatic stellate cells; IP, intra peritoneum; KC, chemokine (C-X-C) ligand 1; MIP-2, macrophage inflammatory protein; α-SMA, alpha smooth muscle actin; TGF-β1, transforming growth factor beta; TUNEL, terminal deoxynucleodidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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ABSTRACT

Liver injury is characterized by hepatocyte apoptosis and collagen producing activated stellate cells (HSC). Hepatocyte apoptosis promotes liver injury and fibrosis whereas activated HSC apoptosis limits hepatic fibrosis. Pharmacologic inhibition of liver cell apoptosis may potentially attenuate liver injury and fibrosis by blocking hepatocyte apoptosis or promote fibrosis by permitting accumulation of activated HSC. To ascertain the net effect of inhibiting liver cell apoptosis on liver injury, inflammation and hepatic fibrogenesis, we examined the effect of a pan-caspase inhibition IDN-6556 on these parameters in the bile duct ligated (BDL) mouse. Hepatocyte apoptosis was assessed by the TUNEL assay and immunofluorescence for active caspases 3/7, and liver injury by histopathology and serum ALT determinations. Real time PCR was employed to measure mRNA transcripts for markers of hepatic inflammation, HSC activation, and fibrosis. Immunohistochemistry for alpha-smooth muscle actin was performed to identify HSC activation. Collagen deposition was quantitated by Sirius red staining and digital imaging techniques. Hepatocyte apoptosis and liver injury (bile infarcts and serum ALT values) were reduced in IDN-6556 -treated vs. saline-treated 3-day BDL mice. Markers for liver inflammation (KC and MIP-2 chemokine expression) and hepatic fibrogenesis (TGF-β and Collagen I expression) were also attenuated. Consistent with these data, HSC activation as

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assessed by alpha-smooth muscle actin mRNA expression and immunohistochemistry was markedly reduced in both 3- and 10-day BDL animals. Collectively, these data suggest hepatocyte apoptosis initiates cascades culminating in liver injury and fibrosis. The pan-caspase inhibitor IDN-6556 is a promising agent for cholestatic liver injury.

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Apoptosis and fibrosis are both ubiquitous features of chronic liver injury (Canbay et al., 2002; Yoon and Gores, 2002). Over time the maladaptive fibrogenic response of the liver to injury culminates in cirrhosis, a pathologic condition characterized by an interconnecting web of dense fibrous strands comprised of collagen. Cirrhosis, when advanced, leads to several deleterious sequela of chronic liver disease, liver failure, and premature death or need for liver transplantation (Kim et al., 2002). Collagen I, the principal collagen responsible for cirrhosis, is generated within the liver by activated stellate cells (HSC), a resident perisinusoidal cell of the liver. These cells are normally quiescent fat storing cells but undergo activation to a myofibroblast like phenotype during liver injury generating collagen I (Friedman, 2000). Apoptosis has been linked to fibrosis by two opposing processes. Hepatocyte apoptosis by death receptors appears to be pro-fibrogenic. For example, genetic or siRNA ablation of Fas expression, a death receptor richly expressed in the liver, attenuates fibrosis in murine models of liver injury (Canbay et al., 2002; Song et al., 2003). In contrast, HSC apoptosis is thought to be essential for the resolution phase of fibrosis (Iredale et al., 1998; Iredale, 2001; Issa et al., 2001). Activated HSC appear to undergo an activation associated cell death terminating their life span and limiting hepatic fibrogenesis. Inhibition of HSC apoptosis would be expected to be pro-fibrogenic by permitting the accumulation of these activated cells within the liver.

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Therefore, the net effect of broadly inhibiting liver cell apoptosis is unclear; it may be ant-fibrogenic by blocking hepatocyte apoptosis or pro-fibrogenic by inhibiting HSC apoptosis. Further, different drugs might have different cellular specificity, however, the net effect of the drug's administration on hepatic fibrosis is probably the most important. This issue needs to be resolved by pre-clinical trials before embarking on human trials with apoptosis inhibitors.

Although inhibition of Fas death receptor-mediated apoptosis reduces hepatic fibrosis, studies cannot be equated with broad inhibition of apoptosis. Death receptors signal through several intracellular cascades, many of which are pro-inflammatory rather than death producing. For example, activation of NF-κB by death receptors is pro-inflammatory (Jaeschke et al., 1998). Consistent with this concept, Fas-activation in the liver is pro-inflammatory and triggers the production of several inflammatory chemokines (Faouzi et al., 2001). The resulting inflammation from Fas activation may result in HSC activation and fibrosis (Maher, 2001). In this context, blocking Fas stimulation may prevent hepatic fibrosis by blocking inflammation, not apoptosis. Therefore the elegant studies examining the relationship between Fas expression and hepatic fibrosis do not directly address the question as to whether broad-based antiapoptotic therapy would be pro- or anti-fibrogenic. Given the complexity of the

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relationships between apoptosis, inflammation and fibrosis, it is important that the *in toto* effects are evaluated experimentally, and then clinically.

Apoptosis is executed by a family of intracellular proteases, referred to as caspases (Thornberry, 1998; Thornberry and Lazebnik, 1998). Although 13 mammalian caspases have been cloned and differ structurally, all caspases cleave on the carboxyl side of aspartate residues. Furthermore, these proteases, which are synthesized as zymogens, are themselves activated by cleavage at aspartate sites (Shi, 2002). Thus, caspase activation requires caspase activity. Because these common characteristics are shared by all caspases, it is possible to synthesize broad spectrum caspase inhibitors which are very effective in blocking cell death (Hoglen et al., 2001; Natori et al., 2003; Valentino et al., 2003). The use of a pan-caspase pharmacologic inhibitor could, therefore, be employed to ascertain if blocking liver cell apoptosis is pro- or anti-fibrogenic.

The aims of the current study were to examine the effects of the pan-caspase inhibitor IDN-6556 on liver injury and fibrogenesis. This drug is the first pan-caspase inhibitor of apoptosis to enter clinical trials (Valentino et al., 2003). The bile duct ligated (BDL) mouse was employed for these studies as it duplicates the hepatocyte apoptosis, HSC activation, and liver fibrosis observed in human liver diseases (Miyoshi et al., 1999; Canbay et al., 2002). To address our aim, several questions were formulated. Specifically,

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we asked does the pan-caspase inhibitor: i) reduce hepatocyte apoptosis? ii) reduce hepatic inflammation? iii) promote or inhibit HSC activation? iv) and attenuate or facilitate hepatic fibrosis? The results demonstrate that the pan-caspase inhibitor IDN-6556 reduces hepatocyte apoptosis, liver injury, hepatic inflammation, HSC activation and hepatic fibrosis; this drug would appear to have potential for the treatment of human liver diseases.

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EXPERIMENTAL PROCEDURES

Extrahepatic cholestasis by ligation of the common hepatic duct. The use and the care of the animals were reviewed and approved by the Institutional Animal Care and Use Committee at the Mayo Clinic. C57/BL6 mice 6-8 weeks of age were employed for these studies. Common BDL was performed as previously described by us in detail (Miyoshi et al., 1999). Sham operated mice, used as controls, underwent a laparotomy with exposure, but not ligation of the common bile duct. In selected experiments, mice were treated with the pan-caspase inhibitor. This drug binds irreversibly to activated caspases with a K_i in the low to sub nanomolar concentrations. The binding is selective, as it does not bind to other cysteine or serine enzymes at nanomolar or even low micromolar concentrations (>10μM). IDN-6556 (10mg/kg) in sterile H_2O mannitol phosphate buffer was given i.p. 3 hours after the BDL, and twice a day thereafter. The dosing was based on preliminary data demonstrating that IDN-6556 at doses of 10 mg/kg were required to prevent liver injury in this model. The agent was obtained from Idun Pharmaceuticals, Inc., (San Diego, CA).

Histology and the TUNEL assay, and immunofluorescence for active caspase 3 and 7. The liver sections, were fixed in 4% paraformaldehyde for 48 hours, and then embedded in paraffin (Curtin Matheson Scientific Inc., Houston, TX). Tissue sections

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(4µm) were prepared using a microtome (Reichert Scientific Instruments, Buffalo, NY) and placed on glass slides. Hematoxylin/eosin staining was performed following standard techniques. TUNEL assay was performed using a commercially available kit, following the manufacturer's instructions (In Situ Cell Death Detection Kit; Roche Diagnostics, Indianapolis, IN). Hepatocyte apoptosis in liver sections was quantitated by counting the number of TUNEL-positive cells in 30 random microscopic low-power fields (630X) as previously described (Miyoshi et al., 1999). Immunofluorescence for active caspase 3 and 7 was preformed as previously described (Natori et al., 2001). Briefly, tissue sections were deparaffinized in xylene, rehydrated with ethanol series, and washed 3 times with PBS for 3 min. The tissue sections were blocked for 60 min in 37°C in blocking buffer (5% goat serum, 5% glycerol, 0.004% sodium azide). The specimens were next incubated for 2 hrs with a rabbit polyclonal anti-active caspase 3/7 antibody (1:50) recognizing a common neoepitope shared by activated caspase 3 and 7 (CM1; BD Pharmingen, San Diego, CA). The sections were washed in PBS 3 times for 10 min, and were incubated with the secondary antibody (1:50) fluorescein-isothiocyanate (FITC-) conjugated swine anti rabbit antibody immunoglobulins (Santa Cruz) at 37°C for 45 min. The slides were mounted with ProLong antifade kit as described in manufacture's instructions (Molecular Probes Inc.).

Determination of Serum ALT determination. Serum alanine aminotransferase

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(ALT) determinations were performed using commercially available assay kits following the manufacturer's instructions (Sigma Diagnostics Kit No.505; Sigma Aldrich, St. Louis, MO).

Real time-polymerase chain reaction (PCR). Total RNA was obtained from whole liver using the Trizol Reagent (Invitrogen, Carlsbad, CA). For each RNA sample, a 10 μg-aliquot was reverse-transcribed into cDNA using oligo-dT random primers and MLV (Moloney Murine Leukemia Virus) reverse transcriptase. Real-time PCR was performed using Taq polymerase (Invitrogen) and primers for α-smooth muscle actin (α-SMA), collagen I (COL1A1), transforming growth factor (TGF)-β1, chemokine (C-X-C) ligand 1 (KC), macrophage inflammatory protein (MIP)-2 as previously described by us (Canbay et al., 2002; Canbay et al., 2003b). 18S primers (Ambion, Inc, Austin TX) were used as a control for RNA isolation and integrity. All PCR products were confirmed by gel electrophoresis.

Real-time PCR was performed using the LightCycler (Roche Diagnostics, Mannheim, Germany) and SYBR green as the fluorophore (Molecular Probes, Eugene, OR). The results were expressed as a ratio of product copies/ml to copies/ml of housekeeping gene 18S from the same RNA (respective cDNA) sample and PCR run.

Determination of liver fibrosis. Liver fibrosis was quantified using sirius red.

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Direct red 80 and Fast green FCF (color index 42053) were provided by Sigma-Aldrich Diagnostics. The areas scanned were captured using the Bacus Laboratories Incorporated Slide scanner (BLISS) system (Lombard, IL). Quantitative histomorphometry was evaluated on the individual images (pixel resolution 480 x 752) for the Sirius red chromogen as previously described (Sebo, 1995; Boone et al., 2000; Canbay et al., 2002)

Immunhistochemistry for alpha-SMA: The sections (4μmm thick) were stained for α-SMA using a mouse monoclonal antibody (NeoMarkers, Fremont, CA), which is prediluted by the manufacturer for staining formalin-fixed paraffin-embedded tissues. The sections were incubated with the antibody overnight at 4°C. Negative control slides were incubated with non immune immunoglobulin under the same conditions. Secondary reagents were obtained from the DAKO LSAB2 kit (DAKO, Carpinteria, CA), and 3,3'-diaminobezidine tetrahydochloride (Sigma Chemical Co., St. Louis, MO) was used for visualization. Finally, the tissue was counterstained with hematoxylin.

Statistics. All data represent at least four independent experiments and are expressed as the mean ± SEM unless otherwise indicated. Differences between groups were compared using ANOVA for repeated measures and a *post hoc* Bonferroni test to correct for multiple comparisons. A *p* less than 0.05 ($p < 0.05$) was considered to be statistically significant.

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RESULTS

Is hepatocyte apoptosis attenuated in IDN-6556-treated BDL mice? We first examined the effects of the caspase inhibitor, IDN-6556, on liver cell apoptosis assessed 3 days after BDL. Apoptosis was quantitated using the TUNEL assay as described under the Experimental Procedures section. TUNEL positive cells (low power field) were significantly reduced in IDN-6556-treated mice compared to saline-treated mice (Fig. 1a and b). Immunohistochemistry for activated caspases 3/7 was also performed to biochemically confirm the occurrence of apoptosis and to determine the efficiency of the pan-caspase inhibitor IDN-6556. The number of caspase 3/7 positive cells increased following BDL and quantitatively were similar to that obtained with the TUNEL assay (Fig.1c). Likewise, in BDL animals receiving IDN-6556, the number of caspase 3/7 positive cells were significantly reduced (Fig.1c). These data demonstrate that IDN-6556 does attenuate hepatocyte apoptosis in the 3-day BDL mouse.

Is liver injury reduced in IDN-6556-treated BDL mice? To examine the effects of caspases in mediating liver injury, liver histology and serum ALT values were examined in BDL mice treated with saline or IDN-6556. Histopathologic examination of liver specimens demonstrated bile ductular proliferation, portal edema and mild portal infiltrates

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in saline-treated mice and in IDN-6556-treated mice, indicating similarly ductular response of the liver to large bile duct obstruction in all groups (Fig. 2a). Confluent foci of hepatocyte feathery degeneration due to bile acid cytotoxicity (bile infarcts) were reduced in IDN-6556 treated BDL animals vs. saline-treated, (23±4.9 vs. 4±1, $p<0.01$) (Fig. 2a). Serum ALT values, an index of hepatocellular injury, were also significantly lower in BDL IDN-6556-treated as compared to saline-treated BDL mice (Fig. 2b). These data implicate a pathogenic role for caspase-mediated hepatocyte apoptosis in liver damage due to cholestasis.

Hepatic inflammation is reduced in IDN-6556 treated BDL mouse. Hepatocyte apoptosis can be a pro-inflammatory process in the liver that promotes chemokine generation and neutrophil infiltration (Jaeschke et al., 1998; Lawson et al., 1998; Faouzi et al., 2001; Canbay et al., 2003b). To examine the effects of caspase-dependent hepatocyte apoptosis in mediating liver inflammation, mice were BDL for 3 days. Messenger RNA transcripts of KC and MIP-2, potent neutrophil chemoattractants and markers of hepatic inflammation, were quantitated using real time PCR technology (Luster, 1998). In saline-treated animals BDL for 3 days, the mRNA for these two chemokines were significantly increased as compared to IDN-6556-treated BDL animals. (Fig. 3a and b). These data suggest inflammation occurs in cholestasis and is coupled to caspase-dependent

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liver injury.

Are liver fibrogenesis and HSC activation reduced in IDN-6556 treated BDL mice? To investigate the role of caspase-dependent liver injury in liver fibrogenesis, mRNA was extracted from the livers of 3-day saline-treated and IDN-6556-treated BDL mice. α-SMA messenger RNA transcripts, an established marker for HSC activation, were quantitated using real time PCR technology, as described in the Experimental Procedures section. In saline-treated 3-day BDL animals, α-SMA mRNA transcripts were significantly increased as compared to sham operated controls, indicating HSC activation in cholestasis (Fig. 4a). In contrast, the transcripts for α-SMA were significantly reduced in IDN-6556-treated BDL animals compared to saline-treated BDL mice (Fig. 4a). These results were confirmed at the protein and cellular level by performing α-SMA immunohistochemistry in 10-day BDL animals. This duration of BDL was selected to permit the potential accumulation of activated HSC in animals receiving the active drug. Consistent with the mRNA data, the number of α-SMA positive cells was actually reduced in drug-treated BDL mice compared to saline-treated BDL animals (Fig. 4b). To ascertain if other markers for HSC activation were also reduced in IDN-6556 treated BDL mice, transcripts for molecules implicated in fibrogenesis were quantitated. TGF-β mRNA, a pivotal cytokine in promoting fibrogenesis, was also increased in BDL saline-treated vs.

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IDN-6556-treated BDL mice (Fig. 4c). Likewise, collagen α1(I) mRNA expression, the principal form of collagen in hepatic cirrhosis, was markedly increased in BDL saline-treated vs. IDN-6556-treated BDL mice (Fig. 4d). These data suggest HSC activation in cholestasis is reduced rather than enhanced in the presence of caspase inhibition.

Finally, to determine if liver collagen deposition is reduced in drug-treated animals, mice underwent BDL, or the sham procedure and treated with IDN-6556 i.p. twice a day for 10 days. This duration of BDL is sufficient for the development of septal fibrosis as assessed by classic histopathologic techniques. Hepatic collagen deposition/accumulation was stained using Sirius red (Fig. 5a) and quantitated using digital image analysis (Fig. 5b). Significant collagen staining was already present in 10 days-BDL mice; however, the quantity of collagen was again significantly increased in saline-treated vs. IDN-6556-treated BDL mice (Fig. 5a and b). Collectively, these observations suggest caspase-dependent liver injury during extrahepatic cholestasis is pro-fibrogenic and that inhibition of caspases with a pan-caspase inhibitor diminishes rather than accentuates hepatic fibrogenesis.

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DISCUSSION

The current studies relate caspase inhibition with hepatocyte apoptosis and fibrogenesis in cholestasis. The observations demonstrate that, in the bile duct ligated animal, pharmacological inhibition of caspases reduces: i) hepatocyte apoptosis, serum ALT values and histologic evidence of liver injury; ii) hepatic inflammation; iii) mRNA expression for markers of HSC activation; and iv) collagen expression and deposition. Taken together, these observations suggest a critical role for hepatocyte apoptosis in the initiation of HSC activation and hepatic fibrogenesis during cholestatic liver injury. Each of these observations is discussed in greater detail below.

Hepatocyte apoptosis following BDL in the mouse is mediated, in part, by the death receptors Fas and death receptor 5/tumor necrosis factor related apoptosis inducing ligand (TRAIL)-receptor 2 (Miyoshi et al., 1999; Higuchi et al., 2002). Apoptosis by death receptors is caspase-dependent (Ashkenazi and Dixit, 1998; Hengartner, 2000). Therefore, our current observations demonstrating that IDN-6556 inhibits hepatocyte apoptosis in the BDL-mouse is consistent with these prior observations. Also as observed in this study, prior publications have linked hepatocyte apoptosis with liver injury (e.g., bile infarcts) and elevated ALT values, and a reduction in these parameters by inhibiting apoptosis.

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However, the current observations demonstrate that a pan-caspase inhibitor currently in clinical trials for the treatment of liver disease has a salutary benefit in a cholestatic disease model. Based on these collective data, this class of drugs merits further study in human cholestatic liver disease.

Our study suggests that apoptosis not only is associated with liver injury but is also pro-inflammatory in the liver during cholestasis. In this study, inhibition of apoptosis with the pan-caspase inhibitor reduced hepatic expression of chemokines. Apoptosis may induce inflammation by several mechanisms. First, dysregulated apoptosis in pathologic conditions can disrupt hepatocyte integrity. For example, after experimental induction of apoptosis with Fas-agonists mice develop fulminant hepatic failure, with massive necrosis and inflammation (Ogasawara et al., 1993). Second, death receptor-mediated apoptosis may contribute to liver inflammation, possibly by initiating pro-inflammatory signaling cascades (Chen et al., 1998; Jaeschke et al., 1998; Kurosaka et al., 2001). For example, Fas agonists induce chemokine expression (Faouzi et al., 2001), and hepatocyte apoptosis is a potent stimulus to neutrophil extravasation (Lawson et al., 1998). Consistent with this concept, a role for neutrophils in liver injury following BDL has recently been reported highlighting a potential relationship between apoptosis and inflammation (Gujral et al., 2003). The disposition of apoptotic bodies may also link apoptosis to inflammation in the

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liver. For example, engulfment of neutrophil apoptotic bodies by macrophages and/or Kupffer cells can induce expression of death ligands, especially Fas ligand (Kiener et al., 1997; Kurosaka et al., 2001; Geske et al., 2002; Canbay et al., 2003a), thereby accelerating apoptosis. Finally, caspases may directly contribute to liver inflammation. Caspases 1,4,5 and 11 have all been implicated in pro-inflammatory cascades (Thornberry and Lazebnik, 1998). A pan-caspase inhibitor such as IDN-6556 may also block liver inflammation by inhibiting these caspases, in addition to reducing cellular apoptosis. Thus, likely by a combination of mechanisms, broad spectrum caspase inhibition is salutary against liver inflammation during cholestasis.

Rather than promoting accumulation of activated HSC, the caspase inhibitor actually blocked their activation. The results suggest apoptosis plays an instrumental if not a pivotal role in HSC activation and hepatic fibrogenesis. Without significant HSC activation, the fear that a caspase inhibitor would block apoptosis of activated HSC, thereby promoting fibrosis, is not a concern. The mechanism by which hepatocyte apoptosis results in HSC activation may be direct or indirect. A direct pathway linking hepatocyte apoptosis to HSC activation is stellate cell engulfment of hepatocyte apoptotic bodies as has been documented in vitro (Canbay et al., 2003c). Phagocytosis of apoptotic bodies by HSC results in their activation and promotes collagen expression. An indirect

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mechanism coupling hepatocyte apoptosis to HSC activation is via a secondary inflammatory response. As described above, hepatocyte apoptosis elicits an inflammatory response associated with chemokine expression and neutrophil infiltration (Maher et al., 1997; Lawson et al., 1998; Miwa et al., 1998; Jaeschke, 2002; Canbay et al., 2003b). This inflammatory response has been well established to cause HSC activation (Maher, 2001). In either model, hepatocyte apoptosis is an apical event resulting in liver injury, HSC activation and liver scarring.

In summary, our findings suggest that during extrahepatic cholestasis in the mouse, both liver injury, inflammation, markers of HSC activation, and elevation of indices of hepatic fibrogenesis are, in part, hepatocyte apoptosis-dependent. Inhibition of hepatocyte apoptosis with a selective caspase inhibitor appears to be a viable therapeutic option for cholestatic liver injury, inflammation and fibrosis. These data also implicate a mechanistic link between hepatocyte apoptosis or the inflammatory response to apoptosis and HSC activation. These preclinical studies support the employment of caspase inhibitors in the treatment of cholestatic and potentially other liver diseases. However, the potentially pro-neoplastic consequences of prolonged and potent apoptosis inhibition will need to be considered and thorough monitoring processes put into place.

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ACKNOWLEDGEMENTS

The secretarial assistance of Erin Bungum is gratefully acknowledged. We thank James Tarara for his excellent technical assistance in digital image analysis.

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Figure 1. Hepatocyte apoptosis is attenuated in IDN-6556-treated BDL mice. IDN-6556-treated and saline-treated mice underwent a sham operation (control) or ligation of their common bile duct. Three days after the surgical procedure, the mice were anesthetized and, liver tissue and serum were obtained. Apoptosis was quantitated using the TUNEL assay and immunofluorescence for active caspase 3 and 7 performed as described under the Experimental Procedures section. (Fig. 1a, b and c) TUNEL and active caspase 3 and 7 positive cells/field were significantly reduced in IDN-6556-treated mice compared to saline-treated BDL mice (P<0.001, n=4 for each group).

Figure 2. Liver injury is reduced in IDN-6556-treated BDL mice. Three days after the surgical procedure, the mice were anesthetized and, liver tissue and serum were obtained. (Fig. 2a) Fixed liver specimens from all mice were stained by conventional H&E. Bile duct proliferation, portal edema and mild portal infiltrates, all features of extrahepatic cholestasis, were present in all BDL mice. However, more bile infarcts (arrows), due to bile acid toxicity, occurred predominantly in saline-treated BDL mice. (Fig. 2b) Serum ALT values are significantly greater in saline-treated than in IDN-6556-treated BDL mice (P<0.005, n=4 for each experimental group)

Figure 3. Hepatic chemokine expression is reduced in IDN-6556 treated BDL mouse. To examine the effects of caspase-dependent hepatocyte apoptosis in mediating

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liver inflammation, mice were BDL for 3 days. KC and MIP-2 chemokine messenger RNA transcripts, marker of hepatic inflammation, were quantitated using real time PCR technology. (Fig.3 a and b) In saline-treated animals BDL for 3 days, the mRNA for these two transcripts were significantly increased as compared to IDN-6556-treated BDL animals (P<0.001, n≥4 for each group). The expression was normalized as a ratio using 18S mRNA as a housekeeping RNA. A value of one for this ratio was arbitrarily assigned to the data obtained from sham operated saline-treated mice.

Figure 4. Markers for HSC activation are increased in untreated compared to IDN-6556-treated BDL mice. Three days after the surgical procedure, liver tissue was procured and total hepatic RNA was isolated as described under the Experimental Procedures section. Alpha-smooth muscle actin (α-SMA), transforming growth factor-β (TGF-β1) and collagen 1α (I) (COL1A1) were quantitated by real-time PCR. The expression was normalized as a ratio using 18S mRNA as housekeeping RNA. A value of one for this ratio was arbitrarily assigned to the data obtained from sham operated saline-treated mice. (Fig. 4a) α-SMA mRNA expression in saline-treated BDL was significantly greater in IDN-6556-treated BDL mice (P<0.001, n=4 for each group). (Fig.4b) Immunoreactivity for α-SMA was increased in saline-treated compared with IDN-6556-treated 10-day BDL mice. The immunoreactivity was sinusoidal in location

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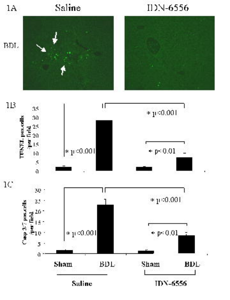
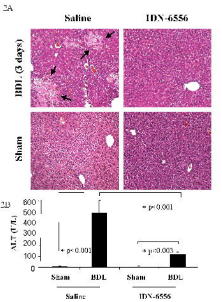
consistent with staining of HSC or myofibroblasts. Indeed, liver sections from saline-treated and IDN-6556-treated sham operated mice displayed little to no α-SMA immunoreactivity (Original magnification 20x). (Fig. 4c) The expression of TGF-β1 mRNA was greater in saline-treated than IDN-6556-treated BDL mice (P<0.005, n=4 for each group). (Fig. 4d) The expression of collagen 1α (I) mRNA was also significantly elevated in saline-treated BDL mice and attenuated in IDN-6556-treated BDL mice (P<0.001, n=4 for each group).

Figure 5. Hepatic fibrosis is reduced in IDN-6556-treated mice as compared to untreated BDL mice. (Fig. 5a) 10 days after the surgical procedure, liver tissue was obtained from BDL and sham-operated saline-treated and IDN-6556-treated mice. Collagen fibers were stained with Sirius red as described under the Experimental Procedures section. (Fig. 5b) The surface area stained with Sirius red was quantitated using digital image analysis. Sirius red staining was quantitatively greater in saline-treated BDL than in IDN-6556-treated BDL mice (P<0.001, n= 4 for each group). Only minimal Sirius red staining was observed in sham-operated mice from the two groups of animals (Original magnification 20x).

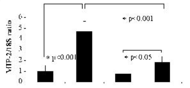


1A
Saline
IDN-6556
BDL
1B
TUNEL pos.cells per field
* p<0.001
* p<0.001
* p<0.01
1C
Casp 3/7 pos.cells per field
* p<0.001
* p<0.001
* p<0.01
Sham
BDL
Sham
BDL
Saline
IDN-6556



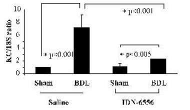
2A
Saline
IDN-6556
BDL (3 days)
Sham
2B
ALT (U/L)
600
500
400
300
200
100
0
* p< 0.001
* p< 0.001
* p< 0.003
Sham
BDL
Sham
BDL
Saline
IDN-6556

3A



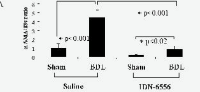
MIP-2/18S ratio
6
5
4
3
2
1
0
* p<0.001
* p<0.001
* p<0.05

3B

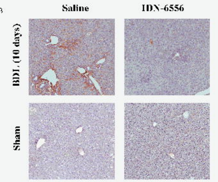


KC/18S ratio
10
8
6
4
2
0
* p<0.001
* p<0.001
* p<0.005
Sham
BDL
Sham
BDL
Saline
IDN-6556

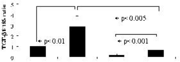
4A


α SMA/18s ratio
6
5
4
3
2
1
0
* p< 0.001
* p< 0.001
* p<0.02
Sham
BDL
Sham
BDL
Saline
IDN-6556

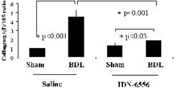
4B


Saline
IDN-6556
BDL (10 days)
Sham

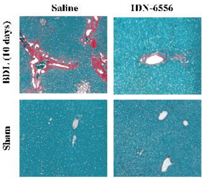
4C


TGF-β1:18S ratio
5
4
3
2
1
0
* p<0.005
* p<0.01
* p<0.001

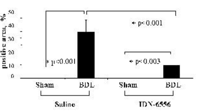
4D


Collagen α1(I):18S ratio
6
5
4
3
2
1
0
* p<0.001
* p<0.001
* p<0.03
Sham
BDL
Sham
BDL
Saline
IDN-6556

5A


Saline
IDN-6556
BDL (10 days)
Sham

5B


Sirius red positive area, %
50
40
30
20
10
0
* p<0.001
* p<0.001
* p<0.003
Sham
BDL
Sham
BDL
Saline
IDN-6556