

# A Caspase Inhibitor, IDN-6556, Ameliorates Early Hepatic Injury in an Ex Vivo Rat Model of Warm and Cold Ischemia

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This study examined the efficacy of the caspase inhibitor, IDN-6556, in a rat model of liver ischemia-reperfusion injury. Livers from male Sprague-Dawley rats were reperfused for 120 minutes after 24 hours of 4°C cold storage in University of Wisconsin solution. Portal blood flow measurements estimated sinusoidal resistance, and bile production, alanine aminotransferase activities, and Suzuki scores were evaluated as parameters of hepatocyte/liver injury. Treated livers were exposed to 25 or 50  $\mu$ M of IDN-6556 in University of Wisconsin storage solution and/or the perfusate. All treatment regimens with IDN-6556 significantly improved portal blood flow measured at 120 minutes, and significant improvements were seen as early as 30 minutes when inhibitor was also present in the perfusate ( $P < 0.01$ ). All treatment groups with IDN-6556 significantly increased bile production by 3-4-fold compared with controls ( $P < 0.01$ ), and reductions in alanine aminotransferase activities were seen within 90 minutes of reperfusion ( $P < 0.05$ ). These data were confirmed by improved Suzuki scores (less sinusoidal congestion, necrosis, and vacuolization) in all treated groups. Livers from the IDN-6556-treated groups had markedly reduced caspase activities and TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling)-positive cells, suggesting reductions in apoptosis. IDN-6556 present in cold storage media ameliorated liver injury due to cold ischemia and reperfusion injury and may be a rational therapeutic approach to reduce the risk of liver ischemia in the clinical setting. *Liver Transpl* 13: 361-366, 2007. © 2007 AASLD.

Received June 9, 2006; accepted September 22, 2006.

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Ischemia and reperfusion injury (IRI) to the liver remains an important problem in clinical medicine, including liver transplantation (LT). Sinusoidal endothelial cells (SEC) appear to be the most sensitive cells in the liver to IRI, with morphological changes occurring early after reperfusion.<sup>1-3</sup> Along with injury/loss of SEC, Kupffer cell activation occurs, resulting in the release of potentially damaging cytokines and reactive species.<sup>4,5</sup> These events, in part, lead to microvascular disturbances, including increased sinusoidal perfusion

pressures and no-flow regions that ultimately cause liver dysfunction and failure, as has been demonstrated in both animal models and humans.<sup>6,7</sup> Because SEC loss may be a major part of graft dysfunction after IRI, and because apoptosis, or programmed cell death, is a major cause of SEC loss after IRI, limiting apoptosis may provide one therapeutic approach to hepatic IRI.<sup>8-10</sup>

A hallmark of apoptosis is the activation of caspases, a family of cysteinyl aspartyl proteases that target critical cellular components.<sup>11</sup> Previous work demonstrated that the caspase inhibitors IDN-1965 and Z-Asp-cmk reduce the number of apoptotic SEC after

**Abbreviations:** ALT, alanine aminotransferase; IRI, ischemia and reperfusion injury; IRLPA, isolated rat liver perfusion apparatus; LT, liver transplantation; SEC, sinusoidal endothelial cells; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; UW, University of Wisconsin solution.

Supported by Idun Pharmaceuticals and JoAnn Barr Foundation.

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DOI 10.1002/lt.21016

Published online in Wiley InterScience (www.interscience.wiley.com).

reperfusion in normothermic and cold ischemia models.<sup>12-14</sup> IDN-6556 is an irreversible pan-caspase inhibitor currently being evaluated clinically in a number of liver diseases, including LT. Pharmacokinetic studies in rats reveal that intravenous administration of IDN-6556 is associated with a half-life of  $51 \pm 11$  minutes and is excreted through the bile.<sup>15</sup> By use of a rat liver cold ischemia and reperfusion injury (IRI) model, Natori and colleagues<sup>16</sup> administered IDN-6556 at a concentration of  $25 \mu\text{M}$  in the preservation solution for 24 hours, followed by ex vivo perfusion. This protocol led to marked reduction in SEC apoptosis as measured by TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) staining and SEC caspase 3 activity. By means of anti-fatty acid synthetase (FAS) and D-galactosamine/lipopolysaccharide models of liver injury that are characterized by hepatic apoptosis, Hoglen and associates<sup>15</sup> reported that intraperitoneal administration of IDN-6556 markedly reduced serum alanine aminotransferase (ALT) levels, hepatocyte apoptosis, and caspase 3 levels. Last, by use of a bile duct ligation model of apoptosis and fibrosis, Canbay and associates<sup>17</sup> reported that administration of IDN-6556 reduced hepatocyte apoptosis and caspase 3/7-positive cells, hepatic necrosis, serum ALT levels, and hepatic inflammation.

However, to our knowledge, there are no published studies that examine whether caspase inhibition with IDN-6556 improves liver function after IRI. Therefore, the purpose of this study was to determine whether inhibition of caspases in a cold ischemia-warm reperfusion model would improve liver function. To accomplish this analysis, we chose cold ischemia followed by warm reperfusion with an ex vivo isolated liver apparatus used as a model, which enabled direct measurement of hepatic sinusoidal pressures and bile production, both of which are directly affected by apoptosis. This study demonstrated for the first time that caspase inhibition by IDN-6556 is associated with improved liver function and diminished liver injury. Collectively, these data provide further evidence that reduced apoptosis through caspase inhibition may ameliorate hepatic IRI.

## MATERIALS AND METHODS

### Animals

Institutional review board approval for this study was obtained before initiation of experiments. Male Sprague-Dawley (Harlan Sprague-Dawley, Indianapolis, IN) rats weighing 250-300 g were used for all experiments. They were cared for according to the guidelines of the University of California at Los Angeles Institutional Animal Research Committee and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were allowed water ad libitum and access to standard rat chow until 12 hours before the experiment. Rats were anesthetized with isoflurane, and all surgical procedures were performed in an aseptic manner. An experimental rat model of hepatic IRI, ex vivo cold ischemia and warm reperfusion, was used in

this study to examine the hemodynamic, biochemical, and histologic manifestations of liver injury.

### Ex Vivo Cold Ischemia and Warm Reperfusion Model

After systemic heparinization, rats underwent transverse laparotomy and cannulation of the portal vein, common bile duct, and inferior vena cava. The livers were flushed through the portal vein with 10 mL of University of Wisconsin (UW) solution (DuPont Pharmaceuticals, Wilmington, DE), procured, and stored for 24 hours at  $4^{\circ}\text{C}$  in UW solution. Livers were then perfused ex vivo for 2 hours on an isolated rat liver perfusion apparatus (IRLPA), as previously described.<sup>18</sup> Temperature was maintained at  $37^{\circ}\text{C}$ , pH of the perfusate was buffered at 7.4, and portal flow was adjusted to keep portal pressure constant at 13 cm  $\text{H}_2\text{O}$ . Portal blood flow recorded at 30-minute intervals was expressed as milliliters per minute per gram of wet liver tissue. Perfusate was collected at 30-minute intervals for determination of ALT, and total bile production was measured at the end of the perfusion time period.

At the conclusion of the experiment, a portion of the liver was frozen in liquid nitrogen for determination of caspase activity, and the remainder of the tissue was fixed in 10% buffered formalin for histopathological and TUNEL analysis. Three treatment groups were compared with untreated control livers ( $n = 4$ ) in these experiments. The first treatment group ( $n = 4$ ) was exposed to  $25 \mu\text{M}$  of IDN-6556 (the gift of Idun Pharmaceuticals, San Diego, CA) in UW solution ( $25 \mu\text{M}$  group) during ischemic storage at  $4^{\circ}\text{C}$ . The second group ( $n = 4$ ) was exposed to IDN-6556 at  $25 \mu\text{M}$  ( $25/25 \mu\text{M}$  group) during both cold ischemia and the 2 hours' reperfusion. The third group ( $n = 4$ ) was exposed to  $50 \mu\text{M}$  of IDN-6556 during both ischemia and reperfusion ( $50/50 \mu\text{M}$  group).

### Syngeneic Liver Transplant Model

The effect of caspase inhibition on survival was determined in a syngeneic liver transplant model between inbred Sprague-Dawley rats. Livers from donor rats were nonrandomly procured and stored with ( $n = 10$ ) or without ( $n = 20$ )  $25 \mu\text{M}$  of IDN-6556 in UW at  $4^{\circ}\text{C}$  for 24 hours and transplanted orthotopically into hepatectomized recipients by the cuff technique.<sup>19</sup> Rats were then observed daily and killed at signs of inanition. Survival was assessed at 7 days.

### Caspase Activity Assay

Frozen tissues from the ex vivo perfusion studies were homogenized in ice-cold hypotonic buffer with protease inhibitors and centrifuged at  $12,000 \times g$  for 15 minutes at  $4^{\circ}\text{C}$  as previously described.<sup>15</sup> Protein concentrations in the supernatant were determined by using the bicinchoninic acid method (Pierce, Rockford, IL), and samples were assayed for caspase activities by monitoring the fluorescent product of the cleavage of DEVD-

amc. The substrate cleavage activity was measured at time 0 and every 30 minutes thereafter for 2 hours in a fluorescent plate reader (excitation wavelength 360/40 nm; emission wavelength 460/40 nm).

### Histological and TUNEL Analysis

Tissue specimens from the ex vivo perfusion studies were stored in 10% buffered formalin and then embedded in paraffin. Sections were cut at a thickness of 4  $\mu$ m, stained with hematoxylin and eosin, and examined and graded in a blinded fashion. Histological injury was blindly graded by a single pathologist (C.L.) with a scoring system from 0 (none) to 4 (severe) according to Suzuki's criteria,<sup>20</sup> which accounts for 3 separate criteria: vascular congestion, hepatocyte vacuolization, and necrosis. The scores for each of the 3 criteria were then combined for a total histological grade of ischemia-reperfusion injury, leaving a combined histopathological score ranging from 0 (normal) to 12 (severe). TUNEL analysis was performed on 4- $\mu$ m sections according to the manufacturer's specifications (Promega, Madison, WI).

### Statistical Analysis

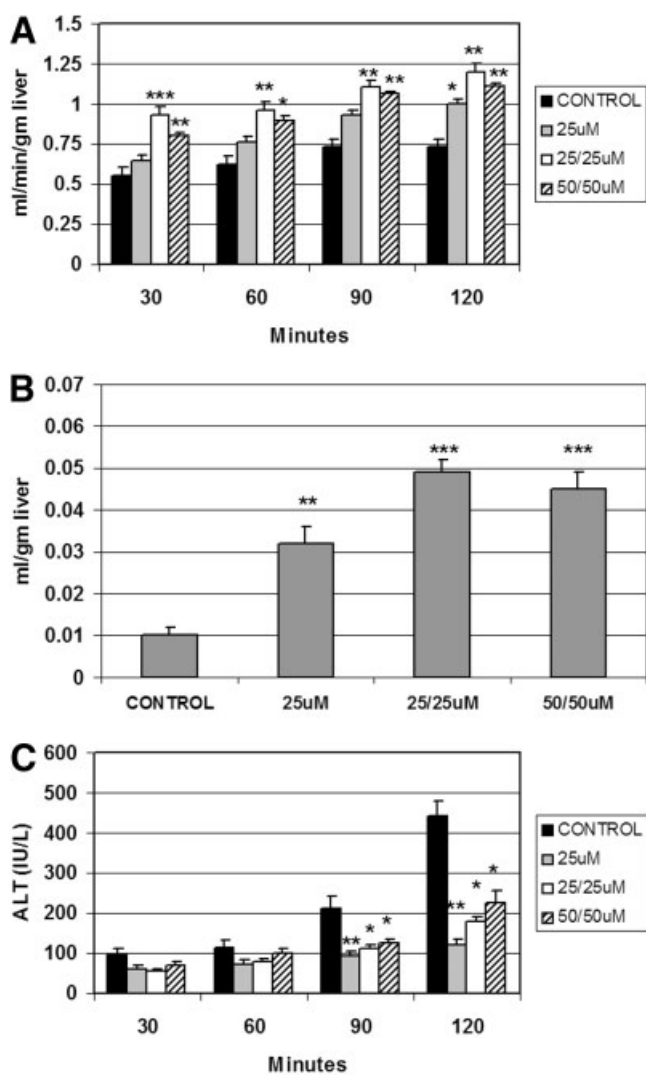
All data are expressed as mean  $\pm$  SEM. Statistical comparisons in the ex vivo reperfusion model were analyzed by 1-way analysis of variance followed by the Bonferroni post hoc test to determine any differences between groups. A *P* value less than 0.05 was considered to be statistically significant, and levels of significance are indicated in the figures.

## RESULTS

### Ex Vivo Cold Ischemia and Warm Reperfusion Model

To evaluate the effects of irreversible inhibition of caspases on hepatic IRI, portal hemodynamics, bile production, ALT release, liver histopathology, apoptosis, and caspase, 3 activities were compared between control livers and those exposed to IDN-6556. While keeping portal inflow pressure at a constant 13 cm H<sub>2</sub>O, portal blood flow (adjusted for liver weight) was measured as an index of sinusoidal resistance. As shown in Figure 1A, the presence of IDN-6556 during both ischemic storage and reperfusion (25/25  $\mu$ M group and 50/50  $\mu$ M group) significantly improved portal blood flow at all time-points studied—30, 60, 90, and 120 minutes (*P* < 0.01). Livers treated with IDN-6556 during only the ischemic storage period (25  $\mu$ M group) showed a significant improvement in portal hemodynamics, but only at 120 minutes of reperfusion as compared with untreated ischemia-reperfusion controls (0.73  $\pm$  0.07 vs. 1.01  $\pm$  0.07, *P* < 0.05).

Total bile production after 120 minutes of warm reperfusion ex vivo was also monitored as a marker of hepatic function. Cumulative bile production was markedly improved in all treated groups exposed to IDN-6556 vs. untreated control livers (Fig. 1B). Bile



**Figure 1. Improvement of (A) portal blood flow, (B) total bile production, and (C) alanine aminotransferase (ALT) by IDN-6556.** The portal vein, common bile duct, and inferior vena cava from hepatectomized livers were cannulated, then perfused and stored in University of Wisconsin (UW) solution for 24 hours with or without addition of IDN-6556 at a concentration of 25 or 50  $\mu$ M. Livers were then perfused ex vivo for 2 hours on an isolated rat liver perfusion apparatus. Temperature was maintained at 37°C, pH was buffered at 7.4, and portal flow was adjusted to keep portal pressure constant at 13 cm H<sub>2</sub>O. Treatment groups: control group, no addition of drug; 25  $\mu$ M group, IDN-6556 present in the UW solution for 24 hours; 25/25  $\mu$ M, IDN-6556 present during cold storage and reperfusion (25  $\mu$ M); 50/50  $\mu$ M, IDN-6556 present during cold storage reperfusion (50  $\mu$ M). Portal blood flow was recorded at 30-minute intervals (mL/min/g wet liver tissue). Total bile produced was collected and quantified over the 2-hour reperfusion period. ALT levels were recorded at 30-minute intervals (IU/L). N = 4-5 per group; data are expressed as mean  $\pm$  SE. \* *P* < .05, \*\* *P* < .01, \*\*\* *P* < .001.

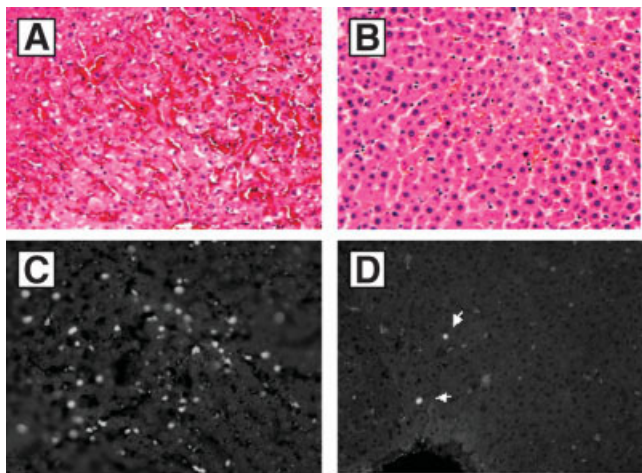
production was 3.3-, 5.1-, and 4.4-fold greater than that of untreated controls for the 25, 25/25, and 50/50  $\mu$ M groups, respectively (*P* < 0.01 to *P* < 0.001). There was no statistical difference between any of the drug-treated groups.

ALT activities in the perfusate of control livers in-

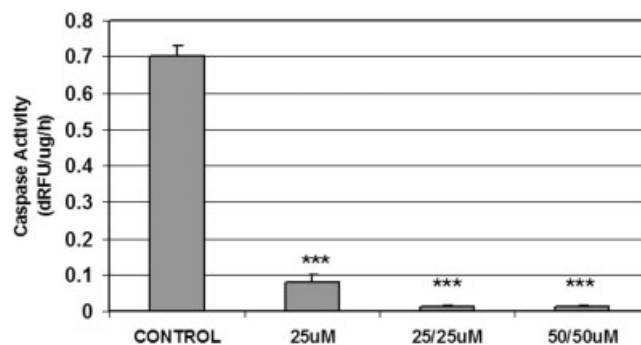
creased steadily from  $90.0 \pm 20$  IU/L at 30 minutes to a peak of  $422 \pm 68$  IU/L by 120 minutes (Fig. 1C), indicating marked hepatocyte injury after perfusion. All three IDN-6556 treatment groups had diminished ALT activities in the perfusate compared with controls. At the 30- and 60-minute time points, although IDN-6556-treated livers had reduced ALT levels, these differences were not statistically significant when compared with controls. However, statistically significant reduction in ALT levels in the 3 treated groups were seen at 90 and 120 minutes as compared with controls. Interestingly, the 25  $\mu$ M group had the greatest reduction in ALT levels, although comparison between the IDN-6556-treated groups did not reveal any statistically significant differences.

Histopathological analysis of liver tissue procured at the conclusion of ex vivo perfusion also revealed differences between treated and untreated livers. Untreated controls showed extensive hepatocyte vacuolization, moderate to severe vascular congestion, and hepatocellular necrosis (Suzuki score  $6.3 \pm 1.0$ ). In contrast, livers treated with IDN-6556 at all concentrations showed a relatively well-preserved sinusoidal architecture with only minimal congestion and necrosis (Suzuki score  $4.8 \pm 0.6$ ,  $5.0 \pm 0.3$ ,  $4.8 \pm 0.6$ , respectively, for the 25, 25/25, and 50/50  $\mu$ M groups). Representative photomicrographs of a control and liver from the 50/50  $\mu$ M treated group are shown (Fig. 2A,B).

To confirm that IDN-6556 prevented apoptosis through caspase inhibition, TUNEL analysis and caspase activities were quantified in liver tissue sections procured after ex vivo perfusion. TUNEL-positive cells were widespread in untreated control livers (Fig. 2C). In contrast, few TUNEL-positive cells were seen in all drug-treated livers, regardless of dose (Fig. 2D). Further, caspase activation was markedly increased in



**Figure 2. Decreased liver injury and TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling)-positive cells by IDN-6556.** Representative hematoxylin and eosin-stained (A, B) and TUNEL-stained (C, D) livers at the conclusion of 2-hour reperfusion ( $n = 4$  per group). For details, see Figure 1. (A) Ischemia and reperfusion injury (IRI) control. (B) IRI liver from the 50/50  $\mu$ M treatment group. (C) IRI control. (D) IRI liver from the 50/50  $\mu$ M treatment group.



**Figure 3. Inhibition of caspase 3 activity by IDN-6556.** At the conclusion of 2-hour reperfusion, livers were snap-frozen and assayed for caspase activity as described in Materials and Methods. For details, see Figure 1. Treatment groups: control group, no addition of drug; 25  $\mu$ M group, IDN-6556 present in University of Wisconsin (UW) solution for 24 hours; 25/25  $\mu$ M, IDN-6556 present during cold storage and reperfusion (25  $\mu$ M); 50/50  $\mu$ M, IDN-6556 present during cold storage reperfusion (50  $\mu$ M).  $N = 4$  per group; data are mean  $\pm$  SE. \*\*\*  $P < .001$ .

control livers ( $0.67 \pm 0.08$ ; Fig. 3), whereas caspase activities were markedly reduced in each IDN-6556 treatment group and was virtually undetectable in the 50/50  $\mu$ M group, indicating maximum inhibition of caspase 3 by IDN-6556.

### Syngeneic Liver Transplant Model

Because the IRLPA analysis demonstrated that IDN-6556 greatly attenuated hepatic injury in the setting of 24 hours of cold IRI followed by reperfusion, we analyzed the survival of livers that were subjected to the same IRI but that were followed instead by LT. Exposure of cold stored livers to the lowest dose of IDN-6556 (25  $\mu$ M) was chosen because it appeared to have similar efficacy, in general, as the other treatment regimens. Control recipients of untreated livers had a 50% 7-day survival, whereas recipients of IDN-6556-treated livers had a 100% 7-day survival ( $P < 0.05$ ). All deaths in the untreated group occurred within 24 hours of LT, and on the basis of necropsy, they were attributed to hepatic dysfunction.

### DISCUSSION

The major aim of this study was to determine whether the pan-caspase inhibitor, IDN-6556, improves functional endpoints after IRI in rat livers. Indeed, the results of this study show that several dosing strategies of IDN-6556 treatment improved the cardinal parameters of IRI. Further analysis confirmed that these improvements were associated with diminished hepatic apoptosis and caspase activities.

Injury of the SEC appears early after reperfusion and is a crucial step in the development of IRI,<sup>4,5</sup> but controversy still remains whether SEC die through apoptosis, necrosis, or both.<sup>21,22</sup> Although necrosis is clearly a form of cell death in both cold and warm reperfusion models, evidence also suggests apoptosis as a domi-

nant form of cell death.<sup>9,10,23</sup> Support for apoptosis as a critical event include several investigations in which treatments targeted against SEC apoptosis have led to reduced IRI.<sup>12,24</sup> There is also evidence in human LT that SEC apoptosis is a prominent feature in histopathology.<sup>8,25</sup>

One of the strengths of this study rests with the advantages obtained through the use of an ex vivo IRLPA. In this model, liver perfusion can be precisely quantified by portal blood flow. This direct perfusion pressure measurement provides a global assessment of liver sinusoidal resistance which is important as one of the presumed mechanisms of action of IDN-6556 is through reduced SEC injury and secondary sinusoidal resistance. This type of direct measurement over this length of time cannot be easily performed by using a LT or in vivo model. Second, the IRLPA provides a direct and facile mechanism for measurement of hepatic bile production. Bile production is an important and sensitive marker of hepatic injury after IRI because marked hepatic injury is commonly associated with diminished bile production.<sup>18,26</sup> A third advantage of the IRLPA is the fact that frequent assay of the hepatic effluent can be performed. This type of frequent sampling is not possible in transplant models because of technical limitation of frequent blood sampling.

Common criticisms of IRLPA models are that the model is ex vivo, bloodless in some instances, and does not provide pulsatile inflow. The IRLPA used in this study negates many of these arguments. First, an enclosed microenvironment is created in which temperature is strictly controlled potentially even better than is seen after LT. Second, the perfusate is a mix of rat whole blood and a buffer thereby achieving the benefit of reperfusion with blood similar to that seen after LT. The portal flow is continuous on the IRLPA as it is after LT. As most rodent LT models do not include arterial reperfusion, the argument for pulsatile inflow is moot. As proof of concept, we extended the ex vivo model into a LT model that incorporated an identical ischemic interval and method and found a marked survival improvement with IDN-6556 treatment.

Although different from prior studies, this investigation complements the existing body of literature regarding apoptosis after liver IRI. Initial studies by Cursio et al.<sup>13,14</sup> examined the effect of a different caspase inhibitor, Z-Asp-2,6-dichlorobenzoyl-oxy-methylketone (Z-Asp-cmk), in a rat in situ warm hepatic IRI model. Treatment with Z-Asp-cmk was associated with improved 7-day survival, histopathology, serum AST and ALT and reduced apoptosis and caspase 3 activity. Several investigations have been reported in liver IRI models using IDN-1965, an earlier and similar molecule to that studied herein. By use of an IRLPA and IDN-1965, Natori et al.<sup>12</sup> found that apoptosis and caspase 3 activity were markedly reduced and that LT survival was improved in the treatment groups. Hoglen et al.<sup>15</sup> investigated the anti-apoptotic effects of IDN-1965 by using an anti-FAS model of hepatic apoptosis. IDN-1965 was very effective at reducing apoptosis as measured by TUNEL staining, histology, and caspase 3 activity.

However, IDN-1965 appeared less effective as a caspase inhibitor when compared with IDN-6556. Natori et al.<sup>16</sup> used a rat IRLPA model of 24 hours IRI to study 6 different caspase inhibitors, including IDN-1965 and IDN-6556. SEC apoptosis was inhibited most strongly by IDN-6556 and was equally effective when added to the cold storage solution alone. Although these results were exciting, the study only examined the apoptotic end points of TUNEL and caspase 3 activity. IDN-6556 was also used successfully in a rat bile duct ligation model of apoptosis/fibrosis.<sup>17</sup>

Therefore, major questions left unanswered before this investigation were whether IDN-6556 was protective in a pure IRI model and whether these protective effects translated into an improved functional outcome. This study confirmed marked inhibition of caspase 3 activities and reduction in TUNEL-positive cells, mainly within the sinusoids after IDN-6556 treatment. Furthermore, this study determined that reductions in caspase activity and apoptosis resulted in considerable improvements in hepatic function. On the bases of the results herein and the existing literature, we hypothesize that reduced SEC apoptosis improves sinusoidal flow during reperfusion, reduces no reflow phenomenon, and leads to a reduction in secondary ischemic insults produced by flow disturbances.<sup>27</sup>

Marked improvements in bile production and ALT activities were also seen, strongly suggesting maintenance of integrity and function of the hepatocyte. Although improvements in bile production have been shown in other IRI models after pharmacological interventions,<sup>26,28</sup> one group of investigators did not note improved bile production after rat LT. However, the caspase inhibitor used in that study differed from the agent used in this study and was specific for caspase 3 only.<sup>29</sup> Additionally, the model used differed substantially.

In conclusion, this study demonstrated that treatment with a pan-caspase inhibitor, IDN-6556, was associated with improved outcome parameters after a severe ischemic insult to the liver. Furthermore, the mechanism by which this effect was achieved appears to involve the direct inhibition of caspases resulting in the diminished SEC apoptosis. These early protective effects provided a more durable protection from the lethality associated with primary hepatic nonfunction after LT. Therefore, caspase inhibition with IDN-6556 is a promising therapeutic modality for the amelioration of hepatic IRI, including that associated with LT.

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