

The Caspase Inhibitor IDN-6556 Prevents Caspase Activation and Apoptosis in Sinusoidal Endothelial Cells During Liver Preservation Injury

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Cold ischemia (CI)–warm reperfusion (WR) liver injury remains a problem in liver transplantation. CI–WR initially causes sinusoidal endothelial cell (SEC) apoptosis through a caspase-dependent mechanism. We previously showed that the caspase inhibitor IDN-1965 prevents CI–WR–induced SEC apoptosis. However, this agent required to be administered to the donor, preservation solution, and recipient for efficacy. Here, we show that a second-generation caspase inhibitor, IDN-6556, effectively prevents CI–WR–induced SEC injury when added only to University of Wisconsin (UW) cold storage media. Rat livers were stored in UW solution for 24 hours at 4°C and reperfused for 1 hour at 37°C. Apoptosis was quantitated using terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay and caspase 3 activation determined by biochemical measurement and immunohistochemical analysis. Pan-caspase inhibitors (IDN-8066, IDN-7503, IDN-7436, IDN-1965, and IDN-6556) were applied at preischemic, cold preservation, or reperfusion periods. TUNEL-positive SEC and caspase 3–like activity in the liver was increased by CI–WR. Three caspase inhibitors (IDN-8066, IDN-1965, and IDN-6556) effectively attenuated SEC apoptosis and caspase 3 activation. The most potent inhibitor, IDN-6556, reduced SEC apoptosis and caspase 3 activity by 55% and 94%, respectively. Prevention of SEC apoptosis by IDN-6556 was not reduced when this agent was administered only during the cold preservation period. When added to the preservation solution, the caspase inhibitor IDN-6556 appears to be a feasible therapeutic agent against ischemia-reperfusion injury in liver transplantation. (*Liver Transpl* 2003;9: 278–284.)

Primary graft nonfunction caused by preservation injury remains a clinical problem in liver transplantation.^{1,2} Moreover, this problem may potentially

increase as non–heart-beating donors, marginal donors, and steatotic livers are used more widely in practice. Although preservation injury is multifactorial, ischemia-reperfusion injury of the liver is an important contributing factor.³ Sinusoidal endothelial cells (SECs) are a cellular target of ischemia-reperfusion injury.^{4–6} SEC injury causes microcirculatory disturbances, including leukocyte migration, platelet activation, and ultimately, secondary injury to hepatocytes leading to organ nonfunction and dysfunction.^{1,6,7} We and others have shown that SEC injury in this process is caused by cell death by apoptosis.^{8–10} SECs show the classic morphological feature of apoptosis after ischemia-reperfusion injury.^{8,9} Biochemical hallmarks of apoptosis, including DNA fragmentation and caspase 3 activation, also are evident. Finally, protease inhibitors known to block apoptosis also protect against liver ischemia-reperfusion injury.^{8,11} Thus, ischemia-reperfusion injury–mediated apoptosis of SECs would appear to be a key feature of liver preservation injury.

The best-characterized proteases contributing to cell injury by apoptosis are the caspases.^{12,13} These proteases are synthesized as proenzymes and activated by proteolytic processing by other caspases.¹² Of caspases cloned and identified to date, caspases 2, 3, 6, 7, 8, 9, and 10 have been implicated most strongly in apoptosis.¹² All these caspases have a cysteine residue in their active site and recognize aspartate in the p1 position of the substrate.¹² This unique substrate specificity has permitted the development of selective caspase inhibitors. Inhibition of caspases by these protease inhibitors often abrogates apoptosis. We recently showed that IDN-1965, a pan-caspase inhibitor, reduced liver injury in a rodent model of ischemia-reperfusion injury relevant to transplantation.⁸ However, for optimal efficacy, the drug had to be included in the University of Wisconsin (UW) storage solution and administered to the donor and recipient. This dosing regimen is complex, cumbersome, and unlikely to be feasible in clinical practice. A drug that can be added only to the UW solution would be most optimal for adaptation to clinical practice.

Since our studies of IDN-1965, a second-generation

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Table 1. Experimental Groups Divided by Treatment With Caspase Inhibitors

Group	Treatment	Intraperitoneal Injection	UW Solution	Reperfusion
1	None	–	–	–
2	IDN-8066	+	+	+
3	IDN-7503	+	+	+
4	IDN-7436	+	+	+
5	IDN-1965	+	+	+
6	IDN-6556	+	+	+
7	IDN-6556	–	+	+
8	IDN-6556	–	+	–

NOTE. Animals were divided into eight groups. In group 1, livers were preserved in cold UW solution for 24 hours at 4°C and subsequently reperused with oxygen-saturated KRH solution for 1 hour at 37°C. In groups 2 through 6, livers were preserved and reperused as described. Indicated caspase inhibitors were administered to animals 10 minutes before anesthesia (10 mg/kg intraperitoneally) and also added to both UW cold storage solution and KRH reperfusion solution. In group 7, IDN-6556 was added to both the UW solution and KRH solution. In group 8, IDN-6556 was added only to UW solution. All caspase inhibitors were applied to either UW solution or KRH solution at a concentration of 25 $\mu\text{mol/L}$.

of caspase inhibitors has been developed. The overall objective of this study is to determine whether these agents are more effective in blocking ischemia-reperfusion injury–induced apoptosis of SECs. In particular, our aim is to identify an agent that is effective when added to the UW solution alone.

Experimental Procedures

Liver Procurement and Storage

Care and use of the animals for these studies were reviewed and approved by the Institutional Animal Care and Use Committee at the Mayo Clinic. Nonfasting adult male Sprague-Dawley rats weighing 280 to 350 g each were used for these studies. Rats were anesthetized using sodium pentobarbital (50 mg/kg of body weight) by intraperitoneal injection. The peritoneal cavity was opened through a midline incision, and the liver was exposed. The portal vein was cannulated with a 14 G angiocatheter. After securing the catheter with 2-0 silk ligatures, the liver was flushed with 30 mL of UW solution at 4°C. The infrahepatic vena cava was cut to enhance flushing of the liver with UW solution. Next, the thoracic cavity was opened and the suprahepatic vena cava was cannulated with a 14 G angiocatheter. After securing this catheter with 2-0 silk ligatures and ligation of the infrahepatic vena cava, the liver was excised from the body cavity and stored in UW solution at 4°C for 24 hours.

Isolated Perfused Rat Liver

A recirculating isolated perfused rat liver apparatus enclosed in a humidified temperature-controlled cabinet was used for these studies.¹⁴ The liver was perfused through the portal vein catheter with oxygen-saturated Krebs-Ringers–*N*-2-hydroxyethylpiperazine-propanesulfonic acid ([HEPES] KRH) solution at 37°C for 1 hour. Venous return from the liver was

collected through the cannula in the suprahepatic vena cava and recirculated after oxygenation. Portal venous flow was maintained at 30 mL/min with a portal venous pressure of 13 cm H₂O.¹⁴

Experimental Groups

Animals were divided into eight groups (Table 1) with four or more animals each as follows. Group 1 livers were preserved in UW solution for 24 hours at 4°C (cold ischemia [CI]), followed by reperfusion for 1 hour at 37°C (warm reperfusion [WR]) without drug. Livers from groups 2 to 6 were preserved and reperused as described, but five different caspase inhibitors were used (IDN-8066, IDN-7503, IDN-7436, IDN-1965, and IDN-6556, supplied by IDUN Pharmaceuticals, La Jolla, CA). Each caspase inhibitor was administered as an intraperitoneal injection (10 mg/kg) 10 minutes before anesthesia, and the inhibitor also was added to both the UW solution and KRH reperfusion buffer at a concentration of 25 $\mu\text{mol/L}$. In group 7, livers were preserved in UW solution and reperused with KRH buffer, both containing 25 $\mu\text{mol/L}$ of IDN-6556. Group 8 livers were preserved with UW solution containing 25 $\mu\text{mol/L}$ of IDN-6556, but the drug was not administered to the animal or included in the reperfusion media.

Quantitation of Apoptosis by a Fluorescent Terminal Deoxynucleotide Transferase-Mediated Deoxyuridine Triphosphate Nick End Labeling Assay

Apoptotic cells were quantitated by the terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay, which enzymatically labels free 3'-OH ends of DNA with a fluorescently labeled nucleotide.¹⁵ Livers were fixed by perfusion with 200 mL of freshly prepared 4% paraformaldehyde phosphate-buffered saline

(PBS), pH 7.4, at room temperature through a portal vein cannula. After perfusion fixation, livers were cut into small tissue blocks (5-mm thick) and further fixed by immersion in 4% paraformaldehyde-PBS solution overnight at 4°C. Tissue samples were embedded in paraffin and cut to 4- μ m sections using a microtome (Reichert Histo Stat 820; Warner-Lambert Tech Inc, Buffalo, NY). After affixing tissue sections to glass slides, sections were deparaffinized by heating for 30 minutes at 60°C, followed by soaking in an xylene bath for 5 minutes twice at room temperature. Tissue sections were hydrated by transferring the slides through the following solutions for 3 minutes at room temperature: 100% ethanol twice; 96%, 90%, 80%, and 70% ethanol; and double-distilled water.

The TUNEL assay was performed by incubating sections with 20 μ g/mL of proteinase K (Boehringer Mannheim Co, Indianapolis, IN) in 10 mmol/L of Tris/HCl buffer containing 5 mmol/L of EDTA (Sigma Chemical Co, St Louis, MO), pH 7.4, at 37°C for 20 minutes. After rinsing twice with double-distilled water, sections were labeled following the instructions of a commercial kit (In Situ Cell Death Detection Kit, Fluorescein; Boehringer Mannheim Co). Briefly, tissue sections were covered with the TUNEL reaction mixture and incubated in a dark humidified chamber at 37°C for 60 minutes. Slides were rinsed three times with PBS and mounted with a glass coverslip (Microscope Cover Glass; Fisher Scientific, Pittsburgh, PA) and mounting media (Permount; Fisher Chemical, Fair Lawn, NJ). Slides were viewed using a fluorescence microscope (Zeiss Axiovert 35; Carl Zeiss Inc, Thornwood, NY) with excitation and emission wavelengths of 490 and 515 nm, respectively. The total number of TUNEL-positive sinusoidal lining cells was counted in 30 random microscopic fields (original magnification \times 400) in each specimen. Data are expressed as number of TUNEL-positive cells per field.

Immunohistochemistry for Activated Caspase 3

Immunohistochemistry was performed using a rabbit anti-caspase 3 polyclonal antibody (CM1 antibody; supplied by IDUN Pharmaceuticals Inc) that only recognizes the neopeptide of the active protease. Liver tissue was paraffin embedded, cut, deparaffinized, and hydrated as described for the TUNEL procedure. Sections were incubated in blocking buffer (2% bovine serum albumin, 0.2% nonfat milk powder, 2% normal goat serum, and 0.8% TritonX-100 in PBS) for 20 minutes at room temperature. Samples were incubated with the primary antibody at a dilution of 1:2,000 in blocking buffer for 30 minutes at 37°C. After washing with PBS, sections were incubated with the secondary antibody (Texas Red-X goat antirabbit immunoglobulin G; Molecular Probes Inc, Eugene, OR) at a dilution of 1:2,500 for 30 minutes at 37°C. After washing with PBS, samples were mounted and viewed using a fluorescence microscope with excitation and emission wavelengths of 595 and 615 nm, respectively.

Measurement of Cytosolic Caspase 3-Like Activity

The right lobe of the liver was excised and immediately homogenized on ice in a hypotonic buffer containing 25 mmol/L of HEPES, 5 mmol/L of MgCl₂, and 1 mmol/L of egtazic acid, pH 7.5, by 30 strokes using a 7-mL Dounce homogenizer with a tight-fitting pestle. The supernatant was obtained by centrifugation at 150,000g for 1 hour at 4°C in an ultracentrifuge (L8-M Ultracentrifuge; Beckman Instruments, Palo Alto, CA). The supernatant was stored at -70°C for subsequent analysis. Caspase 3-like activity was measured as we have described previously in detail by using a fluorescent substrate.¹⁶

Statistical Analysis

All data are expressed as mean \pm SEM from at least three separate experiments. Differences between groups were analyzed using Student's *t*-test when only two groups were analyzed or analysis of variance for repeated measures if three or more groups were analyzed. When analysis of variance was performed, a post hoc Bonferroni test was used to correct for multiple comparisons. All statistical analyses were performed using Instat Software (GraphPad, San Diego, CA).

Results

Comparison of Effects of Caspase Inhibitors on Ischemia-Reperfusion-Induced SEC Apoptosis

First, we examined and compared six different caspase inhibitors; all initially administered to the animal and included in UW storage and reperfusion media (groups 2 through 6, Table 1). Three of these drugs significantly reduced SEC apoptosis; IDN-8066, IDN-1965, and IDN-6556 (Fig. 1). As previously shown by us, IDN-1965 also reduced SEC apoptosis compared with control. Of interest, despite their ability to inhibit caspases in *in vitro* enzyme assays, neither IDN-7503 nor IDN-7436 blocked SEC apoptosis during CI-WR. The inability of these two agents to reduce SEC apoptosis likely reflects their inability to penetrate into this cell population. Of all agents studied, IDN-6556 was the most effective in inhibiting apoptosis. Therefore, we studied this agent in greater detail.

Application of the Caspase Inhibitor IDN-6556 to UW Storage Solution Effectively Prevents Endothelial Cell Apoptosis by Ischemia-Reperfusion

Our previous study showed that the caspase inhibitor IDN-1965 was effective in reducing ischemia-reperfusion-induced SEC apoptosis⁸; however, maximum reduction was observed only when it was applied to

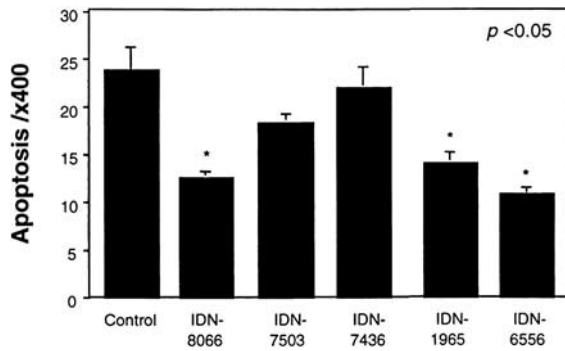


Figure 1. IDN-6556 was the most potent to prevent SEC apoptosis induced by CI-WR. Samples from animals from groups 1 through 6 in Table 1 were analyzed. Apoptotic SECs within the liver were quantitated by TUNEL analysis after 24 hour of CI at 4°C and 1 hour of WR at 37°C. All caspase inhibitors were administered before CI and added to both UW cold storage solution and KRH reperfusion solution. Cells were counted in 30 random fields (original magnification ×400) in each specimen and expressed as apoptotic cells per field. Data expressed as mean ± SEM. * $P < .05$ compared with no caspase inhibitor treatment.

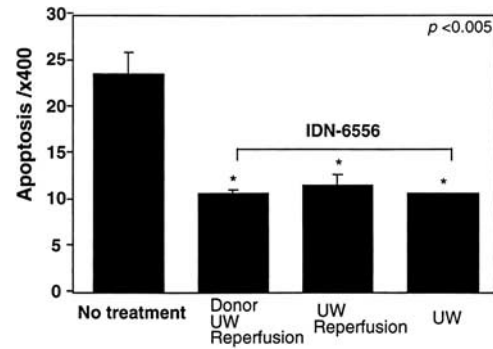


Figure 2. IDN-6556 effectively prevents CI-WR-induced SEC apoptosis when added only to the UW solution. Samples from animals from groups 1 and 6 through 8 in Table 1 were analyzed. Apoptotic SECs were quantitated by TUNEL analysis after 24 hours of CI and 1 hour of WR at 37°C. Caspase inhibitor IDN-6556 was administered to animals before CI (donor) or added to either UW cold storage solution or KRH reperfusion solution. Cells were counted in 30 random fields (original magnification ×400) in each specimen and expressed as apoptotic cells per field. Data expressed as mean ± SEM. * $P < .05$ compared with no caspase inhibitor treatment.

both the donor and UW storage and reperfusion solution.⁸ Because we sought a drug that only needs to be included in the UW storage solution, we next applied the most potent SEC apoptosis inhibitor, IDN-6556, in different application protocols (groups 6 through 8, Table 1). IDN-6556 was equally effective in blocking SEC apoptosis when it was added to the UW solution and reperfusion media (group 8) compared with its addition in only the UW solution (group 9; Fig. 2). These studies quantitated apoptosis by histochemical observations.

However, to provide complementary biochemical information, we measured caspase 3–like activity, a biochemical hallmark of apoptosis. Caspase 3–like activity increased 10-fold after CI-WR compared with normal liver (data not shown). Thus, using a biochemical approach, we were able to confirm the occurrence of apoptosis after liver CI-WR. Caspase 3–like activity was significantly decreased with IDN-6556 treatment compared with livers without IDN-6556 (Fig. 3; $P < .005$). IDN-6556 administration prevented the increase in caspase 3–like activity during CI-WR injury equally when added only to the UW solution and when more broadly applied (Fig. 3). Thus, IDN-6556 effectively blocks apoptosis of SECs when merely included as a component of the UW storage solution.

Caspase 3 Activation Was Predominantly Observed in Sinusoidal Cells, and IDN-6556 Prevents Caspase Activation in This Cell Type

To be sure that IDN-6556 was blocking caspase activation in SECs, we determined the cellular localization of activated caspase 3 by immunohistochemistry using an antibody recognizing the neoepitope of activated

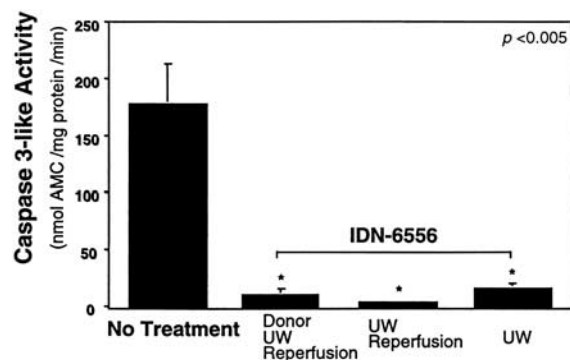


Figure 3. IDN-6556 prevents caspase 3–like activity in rat liver exposed to CI-WR. Samples from animals from groups 1 and 6 through 8 in Table 1 were analyzed. After CI-WR, cytosolic extracts were prepared from livers as described in Experimental Procedures. Caspase 3–like activity was quantitated using the fluorogenic substrate DEVD-MCA. Data expressed as mean ± SEM. * $P < .05$ compared with no caspase inhibitor treatment.

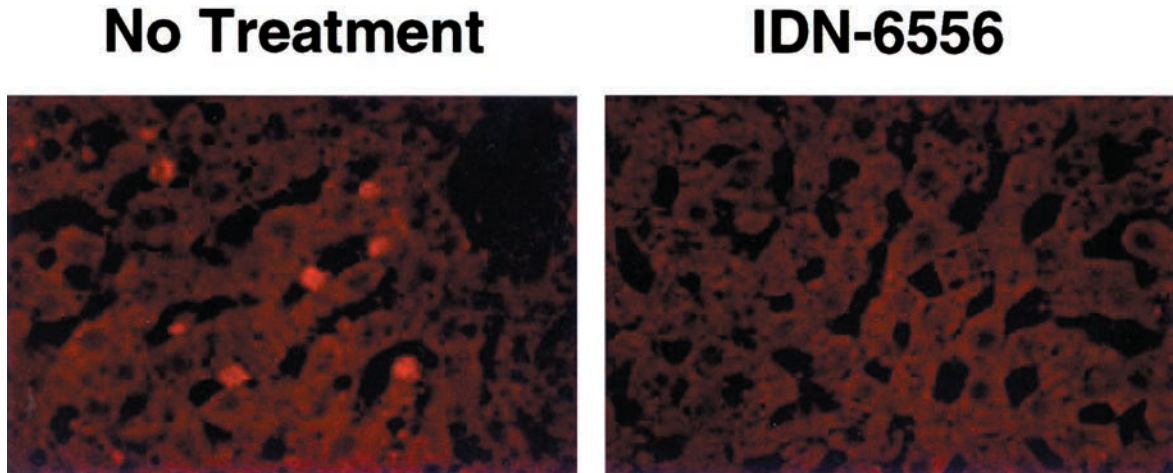


Figure 4. CI-WR induces caspase 3 activation predominantly in SECs, and IDN-6556 prevents caspase 3 activation in SECs. Localization of active caspase 3 was detected by immunohistochemical staining in liver tissue sections. Livers were exposed to CI-WR (left) without caspase inhibitor or (right) with IDN-6556 (25 $\mu\text{mol/L}$ in UW solution). Tissue sections were incubated with a rabbit polyclonal anticaspase 3 antisera that recognizes only the neoepitope of active protease. Immunoreactivity was visualized with a Texas-Red-X labeled antirabbit immunoglobulin G. Note active caspase 3–positive staining was observed only in sinusoidal lining cells, but not hepatocytes. (Original magnification $\times 630$.)

caspase 3. The immunoreactive product was identified only in sinusoidal lining cells after CI-WR injury (Fig. 4). Administration of the caspase inhibitor IDN-6556 to the UW storage solution markedly reduced caspase 3 immunoreactivity in endothelial cells during CI/WR (Fig. 4). Thus, activation of caspase 3 in SECs, the target cells of ischemia-reperfusion injury, was inhibited by administration of IDN-6556.

Discussion

The current studies used a rat liver model of CI-reperfusion injury to examine effects of caspase inhibitors on SEC apoptosis. Although our studies were performed in the rat liver *ex vivo*, SEC apoptosis has been observed *in vivo* in the rat during transplantation paradigms.^{10,17} More importantly, human SEC apoptosis also has been readily identified during hypoxia-reoxygenation injury.^{18,19} Inhibition of SEC apoptosis has been associated with improved organ function, prevention of primary nonfunction, and extended survival in both humans and animals.^{17,20} Thus, the model used for these studies has been well validated and appears, in part, to duplicate human liver CI-reperfusion injury.

Mitochondrial dysfunction with subsequent apoptosis appears to be a key component of liver ischemia-reperfusion injury. Liver reperfusion injury is accompanied by mitochondrial permeability transition,^{21,22} cytochrome *c* release into the cytosol,²³ and activation of certain proapoptotic Bcl-2 family proteins, such as

Bax or Bak.^{24,25} More recently, Selzner et al²⁶ showed that mice overexpressing Bcl-2, an antiapoptotic protein that blocks mitochondrial cytochrome *c* release, are resistant to ischemia/reperfusion liver injury. We previously showed that the mitochondrial membrane potential abruptly dissipates immediately after reoxygenation of anoxic cultured SECs,²⁷ a phenomenon observed in numerous studies involving mitochondrial dysfunction with cytochrome *c* release.^{28,29}

Several observations suggest that oxidative stress caused by either an increase in generation of reactive oxygen species, depletion of antioxidants (e.g., glutathione depletion), or both mediates apoptosis through an alteration in mitochondrial function.^{30–33} SECs may be more susceptible than hepatocytes to oxidative stress because of reduced concentrations of endogenous antioxidants compared with parenchymal cells.^{34,35} Thus, SEC oxidative stress with mitochondrial dysfunction and subsequent activation of the apoptotic program likely is a key component of CI-reperfusion liver injury.

Caspase 3 is activated in the mitochondrial dysfunction pathway of apoptosis after release of cytochrome *c* from the intermembrane space.^{36,37} Cytosolic cytochrome *c* binds apoptosis activating factor 1, facilitating the recruitment of procaspase 9 to this protein complex referred to as the apoptosome.^{38–40} Procaspase 9, activated within the apoptosome, directly activates caspase 3.^{38–40} Caspase 3 is well established as a distal effector protease in apoptosis and will directly induce apoptosis

in cellular or cell-free models.^{36,37,41} Our findings that caspase 3 was activated in SECs during ischemia-reperfusion injury therefore is consistent with activation of the mitochondrial apoptotic pathway in this cell type. These immunohistochemical and biochemical data also confirm biochemically the occurrence of apoptosis of SECs in this model of ischemia-reperfusion injury. Furthermore, the observation that IDN-6556 significantly decreased tissue caspase 3 activity compared with untreated livers strongly suggests that the salutary effect of this agent was caused by its ability to inhibit caspases. Because IDN-6556 is a pan-caspase inhibitor, it may inhibit caspase 3 activity by inhibiting caspase 3 directly or inhibiting the upstream caspase, caspase 9, or both caspases.

Results of the current study complement those of Anselmo et al,⁴² recently published in preliminary form. Also using the rat model of organ preservation, their studies showed that IDN-6556 decreases vascular resistance, increases bile flow, and improves liver function. More importantly, using a syngeneic model of rat liver transplantation with 24 hours of cold ischemic storage in UW solution, animal survival at 7 days was improved from 50% to 100%. These studies, coupled with results reported here, suggest caspase inhibition with IDN-6556 attenuates liver preservation injury.

The occurrence of SEC apoptosis in ischemia-reperfusion injury is dependent on the reperfusion component of this injury. For example, previous studies have shown that 24 hours of cold preservation alone do not elicit SEC apoptosis or caspase 3 activation.^{8,9} Therefore, it would appear that therapeutic maneuvers designed to inhibit SEC apoptosis need to target, at a minimum, the reperfusion phase of ischemia-reperfusion injury. Based on this concept, we reasoned that if a cell permeant caspase inhibitor could be introduced into the organ before reperfusion, SEC apoptosis would be attenuated. Administering an agent in the storage solution rather than to potential donors or the recipient at the time of reperfusion has several advantages. In particular, this strategy minimizes exposure of the drug to other organs that also are procured from the donor, eliminating potential toxicity to these organs. Also, obviating the need to administer the agent to the donor eliminates systemic side effects, drug-drug interactions, and so on, which may complicate the care of the complex and ill transplant recipient.

Of the caspase inhibitors examined, IDN-6556 reduced SEC apoptosis significantly, even when added to only the UW solution. Other caspase inhibitors were not as efficacious as this compound when added only to the UW storage solution. Presumably, the ability of

these inhibitors to penetrate endothelial cells during cold storage was limited or they were easily washed out during reperfusion. Nonetheless, current results suggest that IDN-6556 can be used in UW solution as a therapeutic drug to limit liver ischemia-reperfusion injury.

In conclusion, SEC apoptosis occurs in CI-reperfusion injury through caspase 3-dependent mechanisms. The pan-caspase inhibitor IDN-6556 inhibited caspase 3 activation and reduced SEC apoptosis when used as an additive in the UW storage solution. Thus, this caspase inhibitor appears to provide a pharmacological approach to reduce ischemia-reperfusion injury in liver transplantation. Recent phase I trials have been performed in humans, indicating the drug is safe during short-term administration.⁴³ Collectively, this information suggests that a therapeutic trial of IDN-6556 in liver transplantation to reduce the sequelae of ischemia-reperfusion injury is rational.

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