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Clinical Trial of the Pan-Caspase Inhibitor, IDN-6556, in Human Liver Preservation Injury

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Cold ischemia/warm reperfusion (CI/WR) injury remains a problem in liver transplantation. The aim of the current study was to assess the utility of the pan-caspase inhibitor IDN-6556 on CI/WR injury during human liver transplantation. This report is a *post hoc* analysis of a Phase II, multi-center, randomized, placebo-controlled, double-blinded, parallel group study. Subjects were assigned to four treatment groups: Group 1 (Organ storage/flush: Placebo—Recipient: Placebo); Group 2 (Organ storage/flush: 15 μg/mL—Recipient: Placebo); Group 3 (Organ storage/flush: 5 μg/mL—Recipient: 0.5 mg/kg); and Group 4 (Organ storage/flush: 15 μg/mL—Recipient: 0.5 mg/kg). Liver cell apoptosis was assessed by serum concentrations of the apoptosis-associated CK18Asp396 ('M30') neo-epitope, TUNEL assay and caspase 3/7 immunohistochemistry. Liver injury was assessed by serum AST/ALT determinations. Serum markers of liver cell apoptosis were reduced in all groups receiving drug as compared to placebo. However, TUNEL, caspase 3/7 positive cells and serum AST/ALT levels were only consistently reduced in Group 2 (drug exposed to organ only). This reduction in serum transaminases was significant and observed across the study. In conclusion, IDN-6556 when administered in cold storage and flush solutions during liver transplantation offers local therapeutic protection against CI/WR-mediated apoptosis and injury. However, larger studies are required to confirm these observations.

Key words: Apoptosis, clinical trial, cold ischemia/warm reperfusion injury, neutrophil infiltration

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Introduction

Cold ischemia/warm reperfusion (CI/WR) injury remains a problem in liver transplantation. CI/WR injury causes up to 10% of early organ failure and has been associated with an increased incidence of both acute and chronic rejection (1). The first consequence of ischemic liver injury, which occurs during cold preservation, is tissue anoxia which disturbs intracellular energy metabolism reducing cellular levels of adenosine triphosphate (2). Although reperfusion restores blood flow and oxygen to the liver, terminating the ischemic period, the introduction of oxygenated blood aggravates the initial ischemic insult by promoting the formation of reactive oxygen intermediates (3). Thus, the cellular manifestations of CI/WR injury occur principally during reperfusion, although biochemical changes occur during both phases of injury. There are several components to CI/WR injury. The initial pathophysiologic consequence is liver cell injury, especially microvascular injury. This tissue damage generates secondary insults such as neutrophil infiltration, macrophage/Kupffer cell activation and cytokine generation (4). These secondary events can culminate in microvascular injury, perfusion abnormalities, massive cellular injury and organ failure with fatal consequences.

Increasing evidence points to apoptosis as a critical mechanism during the initial phase of hepatic CI/WR injury in both animal (5,6) and human liver allografts (7,8). Apoptosis, a form of cell death critical in regulating tissue homeostasis is mediated by activation of cysteine proteases referred to as caspases (9). These proteases exist within the cell as zymogens and can be divided into initiator (caspases 2, 8, 9 and 10) and effector caspases (3, 6 and 7). Caspases are key cellular mediators of apoptosis after liver CI/WR (3,10–12).

Recently, IDN-6556 (3-{2-(2-tert-Butyl-phenylaminooxalyl)-amino]-propionylamino}-4-oxo-5-(2,3,5,6-tetrafluoro-phenoxy)-pentanoic acid), a novel irreversible broad-spectrum caspase inhibitor has been shown to reduce CI/WR-induced apoptosis and injury in the liver (13–16) and other

organs (17). In rat experiments (16), administration of IDN-6556 resulted in portal drug concentrations 3-fold higher than systemic concentrations, indicating a significant first-pass effect. Levels remained constant until excreted intact in the bile. A phase I clinical trial of IDN-6556 administered to patients with hepatic impairment showed the drug to be well tolerated. It also contributed to a marked reduction in serum AST/ALT levels (15). Thus, the pan-caspase inhibitor, IDN-6556 is a potential agent for the treatment of human liver injury characterized by excessive apoptosis.

The overall objective of this Phase II clinical trial was to evaluate the effects of IDN-6556 during human liver transplantation. To address this objective, three fundamental questions were formulated: (1) Does IDN-6556 reduce CI/WR-mediated liver cell apoptosis? (2) Is postoperative liver injury ameliorated by treatment with IDN-6556? and (3) Is administration of IDN-6556 well tolerated? The results of this *post hoc* analysis indicate that administration of IDN-6556 in the storage and flush solutions may reduce CI/WR-induced apoptosis, postoperative liver injury and inflammation without serious adverse events. IDN-6556 may represent a promising therapeutic approach for reducing to CI/WR injury during liver transplantation surgery.

Methods

Patient population, eligibility and exclusion criteria

This study was conducted as a multicenter trial, including institutions in the United States and Europe. This study was approved by all Institutional Review Boards. Ninety-nine adult liver transplant recipients were enrolled. Subjects were considered eligible if they were of the minimum adult legal age according to local laws and able to provide written informed consent. Reasons for exclusion were as follows: (a) fulminant hepatic failure (UNOS Status I patients); (b) previous liver transplantation; (c) patients undergoing multiple organ transplantation; (d) patients receiving split liver grafts; (e) extrahepatic malignancy; (f) any patient receiving a living donor allograft; (g) any patient who had received any investigational drug or device within 30 days of study drug administration, or who was scheduled to receive another investigational drug or device within 30 days after study drug administration and (h) any patient who was pregnant, lactating or had a positive serum pregnancy test at preoperative evaluation. All study procedures were in accordance with the Helsinki Declaration of 1975, with the latest amendments Edinburgh, Scotland (2000) and the International Conference on Harmonization guidelines on Good Clinical Practice (Step 4, May 1, 1996).

Study design

The study was registered at www.clinicaltrials.gov identifier: NCT00080236. This study represents a *post hoc* analysis of a Phase II, multicenter, randomized, placebo-controlled, double-blinded, parallel group study. Subjects were assigned to one of four treatment groups on a sequential basis (Table 1). The first group (Group 1) received placebo during donor organ cold storage and flush and placebo was administered to the recipient, while the second group (Group 2) received study drug (IDN-6556) during cold storage and flush and placebo was given to the recipient. For Group 2, IDN-6556 was supplied in the University of Wisconsin (UW) or histidine-tryptophan-ketoglutarate (HTK) solution at a concentration of 15 μg/mL and was perfused through the portal vein prior to organ transplantation. Group 3 received the study drug in the cold storage and flush solutions at a concentration of 5 μg/mL and following liver transplantation, intravenously (0.5 mg/kg) every 6 h for 24 h, and Group 4 received IDN-6556 (15 μg/mL) in the storage and flush solutions and was administered to the recipient at a concentration of 0.5 mg/kg every 6 h for 48 h.

Table 1: Study design

Study design group	Donor organ (storage and flush solutions)	Recipient (i.v.[1] every 6 h × 24 h)
Group 1	Placebo	Placebo
Group 2	IDN-6556 (15 μg/mL)	Placebo
Group 3	IDN-6556 (5 μg/mL)	IDN-6556 (0.5 mg/kg)
Group 4	IDN-6556 (15 μg/mL)	IDN-6556 (0.5 mg/kg)

[1] Intravenously.

Efficacy assessment

Efficacy was assessed from day 1 post liver transplantation to day 7. Primary efficacy endpoints of the study included: (1) Analyses of the course of liver function tests (AST/ALT levels) and other biochemical parameters (international normalized ratio, serum total and direct bilirubin, serum creatinine) by using repeated measurement analysis to evaluate treatment and time effects; (2) peak liver function tests (AST/ALT) over time; (3) donor back-table preperfusion liver biopsy and intraoperative postreperfusion liver biopsy; (4) 7-day post-transplant liver biopsies were obtained from Mayo Clinic, Rochester patients per a preexisting institutional protocol. Seven to 14 day posttransplant biopsies were obtained from San Antonio Health Science Center (SAHSC) and University of California at San Francisco (UCSF) if the clinical scenario warranted hepatic parenchymal evaluation; (5) duration of intensive care unit stay and (6) rates of primary nonfunction (PNF), rejection and retransplantation.

Safety evaluation

Safety was assessed by monitoring patient outcomes and recording systemic adverse events throughout the study from day 1 posttransplant to discharge. All subjects were evaluated for study drug safety and tolerability at the time of transplantation, daily for 7 days, and at 4 months and 1 year post transplant. Systemic adverse events were monitored by physical examination, vital signs, laboratory serum and urine samples and liver biopsy. Liver biopsies were obtained from the donor back-table preperfusion liver, intraoperative postreperfusion liver, and on day 7 (Mayo Clinic, Rochester only), and days 7–14 (SAHSC and UCSF) after liver transplantation as clinically indicated.

Immunosuppression

Patients received standard immunosuppression according to the protocols of the respective institutions. Most protocols consisted initially of tacrolimus, mycophenolic acid and prednisone. The target blood levels of tacrolimus were 10–15 ng/mL in the first 2 weeks and 5–10 ng/mL thereafter. Mycophenolic acid was given as a dose of 2 g/day and discontinued between 2 and 4 months after liver transplantation. The dose of prednisone was tapered and discontinued completely by 4 months after transplantation.

Measurements of apoptosis

M30-Apoptosense® enzyme-linked immunosorbent assay (ELISA) was used to quantitate the measurement of the apoptosis-associated CK18Asp396 ('M30') neo-epitope in serum samples. The M30 antibody recognizes a neo-epitope which is exposed after cleavage of CK18 by caspases after the aspartic acid residue 396 (CK18Asp396) (18). Cleavage at this position occurs early during apoptosis and is mediated by caspase-9 as well

as caspase-3 and -7 (19). Serum samples for ELISA were collected from study participants beginning intraoperatively (after venous perfusion) and prior to each intravenous infusion of study drug during the first day after liver transplantation (IDN-6556 was delivered every 6 h for the first 24 h after transplantation) and stored at –20°C until analyzed. The ELISA assay was performed using a commercially available kit, following the manufacturer's instructions, after verification of manufacturer's performance claims and establishing linearity for higher range samples expected in this study (Peviva AB; Axxora; Bingham, UK) (20). Normal 'M30'value: median = 121.5 U/L as determined by the manufacturer.

TUNEL assay

The TUNEL assay was performed as previously described (21) using the Apoptag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon, Temecula, CA) with minor modifications. Briefly, slides were treated with 20 ug/mL proteinase K at 37°C for 15 min. Following a phosphate buffered saline (PBS) rinse, endogenous peroxidase activity was quenched by incubating the slides in a hydrogen peroxide/methanol solution for 10 min. After washing again in PBS, a 1:5 dilution of terminal deoxynucleotidyl transferase was added and incubated at 37°C for 2 h. After applying stop solution for 15 min and washing, samples were incubated with antidigoxigenin peroxidase conjugate at 37°C for 30 min. Slides were developed with a 1:20 dilution of diaminobenzidine substrate, counterstained with methyl green and rehydrated. Then the number of TUNEL positive cells per high power field (hpf) (40×)/total hepatocytes × 100 was quantitated by a pathologist masked to the study design.

Immunostaining for active caspase-3/7

Formalin-fixed, paraffin-embedded samples underwent heat inactivated epitope recovery using 1 mM EDTA pH 8.0. Sections were subsequently washed in distilled water before being incubated with 3% H_2O_2 in ethanol for 5 min to inactivate the endogenous peroxides. Sections were then incubated with a 1:200 dilution of Anti-Caspase-3/7 (Biocare Medical, Walnut Creek, CA) for 60 min. After rinsing with Tris buffered saline wash buffer plus Tween (TBST), the secondary reagent (rabbit-MACH3 (Biocare Medical) was added and the slides were incubated for 10 min. The slides were again rinsed with TBST wash buffer before being developed in Nova Red (Vector, Burlingame, CA). Slides were subsequently counterstained with hematoxylin. Then the number of caspase 3/7 positive cells per hpf (40×)/total hepatocytes × 100 was quantitated by a pathologist masked to the study design.

Immunohistochemistry for hepatic neutrophils

Unstained liver sections were stained for myeloperoxidase (MPO) using a rabbit polyclonal antibody (NeoMarkers, Fremont, CA), at a dilution of 1:100 as previously described by us (22). Then the number of MPO-positive cells per hpf (40×)/total hepatocytes × 100 was quantitated. A pathologist (masked to the study design) then independently scored the slides from 1 to 3+. The grades correspond to the following percentages: 1–3+ scale: 1+≤20%; 2+ = 20–40% and 3+≥40%. Zonal or nonzonal distribution in the liver of the most MPO-positive cell was also designated.

Statistics

For efficacy studies and the presence of apoptotic markers results were expressed as mean values ± SD. When comparing differences between groups, a Student's *t*-test was performed. For comparisons involving serum parameters (AST/ALT, creatinine, bilirubin) differences between groups were analyzed using all values collected during the first 7 days after liver transplant. Comparisons were also made between groups with data 4 months and 1 year after liver transplant. For comparisons involving 7-day biopsy data, analyses for grade of neutrophil disbursement, Fisher's exact tests were performed with weighted data points. Data were weighted such that 1+ was assigned one point, 2+ was assigned two points and 3+ was assigned three points. Statistical tests were performed at a nominal significance level of 0.05 ($p < 0.05$). No statistical adjustment was considered for multiplicity of statistical testing.

Results

Data were available for all 99 adult liver transplant recipients. All 99 randomized subjects received at least one study treatment and were included in the safety analyses, Group 1 (Organ storage/flush: Placebo—Recipient: Placebo), n = 23, Group 2 (Organ storage/flush: 15 μg/mL—Recipient: Placebo), n = 23, Group 3 (Organ storage/flush: 5 μg/mL—Recipient: 0.5 mg/kg), n = 27 and Group 4 (Organ storage/flush: 15 μg/mL—Recipient: 0.5 mg/kg), n = 26. All 7-day protocol biopsies were performed at one center (Mayo Clinic, Rochester) n = 35/99. Complete data from all study participants were available for the first 7 days and no patients were lost to follow-up. Baseline demographic characteristics were well balanced across all treatment groups (Table 2). The indications for liver transplantation in each study group are listed in Table 2.

Does IDN-6556 reduce apoptosis during ischemia reperfusion injury in human livers after transplantation?

Apoptosis, as determined by quantitation of caspase 3/7 positive cells, was increased in intraoperative postreperfusion liver biopsies (7% ± 0.01) compared to donor backtable preperfusion liver specimens (2% ± 0.02) in Group 1 ($p < 0.03$). Thus, there was a rapid increase in cellular apoptosis during the reperfusion phase of the injury. This increase in apoptosis was prevented in treatment Groups 2, 3 and 4 which demonstrated no significant difference in levels of caspase 3/7 activation from preperfusion to postperfusion biopsies (Figure 1A). Apoptosis was then assessed indirectly over the next 24 h after transplantation by employing the M30-Apoptosense ELISA to quantitate the CK18Asp396 ('M30') neo-epitope in serum samples (Normal 'M30'value: median = 121.5 U/L). Serum samples collected from recipients intraoperatively (after venous perfusion) and prior to intravenous infusion of IDN-6556, had significantly elevated M30 concentrations (>5-fold) above normal median values in all groups. Group 1 (placebo), when compared to other groups (Figure 1B) had the highest serum levels. By the fourth serum draw Groups 1 and 2 showed similar increasing M30 concentrations ($p = 0.11$). In contrast, Groups 3 and 4 M30 levels did not rise and remained significantly lower when compared to Group 1 ($p < 0.0001$ for Group 3) and ($p < 0.001$ for Group 4). Thus, while organ storage with IDN-6556 reduced the initial phase of reperfusion-mediated apoptosis in liver tissue, intravenous drug was necessary to reduce serum apoptosis during the next 24 h.

Interestingly, by day 7, Group 2 had reduced levels of apoptosis as depicted by TUNEL assay (7.6% ± 2.5) and

Table 2: Study demographics and indications for liver transplantation

	Description of the study population			
	Group 1	Group 2	Group 3	Group 4
Number per group (n)	23	23	27	26
Mean age[1] (donor)	45 ± 17.9	45.6 ± 14.3	38.2 ± 17.6	41.3 ± 16.6
Mean age[1] (recipient)	52.7 ± 9.7	54.3 ± 7.8	52.9 ± 11.3	51.5 ± 10.5
% Female (donor)	34.7	47.6	46.1	40.0
% Female (recipient)	13.6	23.0	34.0	36.0
Recipient MELD score	25 ± 7.4	24.4 ± 8.5	26.1 ± 7.7	27.0 ± 8.9
Indications for transplantation				
HBV	2	2	0	1
HCV	8	3	6	3
ASH	1	5	4	5
NASH	1	2	2	3
NET	2	1	1	0
Cryptogenic cirrhosis	3	3	4	3
PBC/PSC	3	3	2	5
HCC	1	3	5	4
CCA	1	0	1	0
Other[2]	1	1	2	2

MELD = model for end-stage liver disease; HBV = hepatitis B cirrhosis; HCV = hepatitis C cirrhosis; ASH = alcoholic steatohepatitis; NASH = nonalcoholic steatohepatitis;, NET = neuroendocrine tumor; PBC/PSC = cryptogenic cirrhosis, primary biliary cirrhosis/primary sclerosing cholangitis; HCC= hepatocellular carcinoma (HCC); CCA = cholangiocarcinoma.
[1]Age in years.
[2] Autoimmune hepatitis, hepatotoxicity, hemochromatosis, anabolic steroid abuse, congenital extrahepatic biliary atresia.

caspase 3/7 immunostaining (8.0% ± 3.0), as compared to Group 1 (TUNEL: 10.3% ± 5; caspase 3/7:14% ± 6). TUNEL and caspase 3/7 positive cells were reduced by day 7 from liver specimens in Group 3 (TUNEL: 7.7% ± 3.3; caspase 3/7: 9.0% ± 8), but to a lesser degree when compared to Group 2. However, Group 4 showed no incremental decrease in TUNEL (9.6% ± 5) or caspase 3/7 positive cells (26% ± 3) on day 7 biopsy. Thus, by 1 week after transplantation, intravenous drug administration actually overturned the salutary effects of including IDN-6556 in the storage solution.

Is postoperative liver injury following transplantation ameliorated by IDN-6556?

The serum transaminases were monitored daily for the first 7 postoperative days, as serum AST and ALT levels are known to be elevated in the immediate postoperative period after liver transplantation secondary to ischemia-reperfusion injury. Serum AST and ALT levels were significantly reduced during postoperative days 1–7 in Group 2 compared to Group 1 (Figure 2A,B) ($p < 0.01$ for AST and $p < 0.01$ for ALT). In contrast, Groups 3 and 4 did not experience a reduction in serum enzyme levels immediately postoperatively, in fact Groups 3 and 4 demonstrated elevated serum levels of AST/ALT similar to the placebo group. All groups reached close to normal range by day 7. Serum creatinine and total bilirubin levels were evaluated at the same time points and no difference was observed between groups, suggesting that IDN-6556 had no adverse effect on renal or biliary function (Figure 2C,D). These data suggest that a pan caspase inhibitor provided to the transplanted organ during cold-ischemic storage but not following reperfusion can reduce liver injury.

Can the occurrence of liver rejection and inflammation be reduced by IDN-6556?

Protocol liver biopsies on postoperative day 7 (Mayo Clinic, Rochester only) (n = 35) and clinically relevant biopsies 7–14 days postoperatively (SAHSC and UCSF only) (n = 33) were obtained for pathologic review. In Group 1 there were 6 patients who developed acute cellular rejection of the 17 total patients randomized to this group. In Group 2 the number of acute episodes of cellular rejection was 4 of 15, and in Group 3 it was 5 of 18 and in Group 4 it was 5 out of 18. Thus, in this limited sample population, IDN-6556 did not alter the rates of acute cellular rejection above that observed in untreated patients.

Table 3 highlights MPO immunohistochemical analysis for neutrophils performed on liver specimens 7 days after transplantation to assess if inflammatory change was responsible for serum AST/ALT and apoptotic differences observed between the groups. Interestingly, liver specimens from Group 2 demonstrate the least amount of MPO stain/hpf as compared to other treatment groups (Table 3). In fact, liver tissue from Group 4 showed significantly increased MPO stain/hpf when compared to Group 2 ($p < 0.04$), and demonstrated a similar hepatic neutrophil immunostain quantitation and distribution as placebo ($p = 0.65$). These data suggest that administration of IDN-6556 in the storage and flush solutions alone may reduce neutrophil infiltration of the liver in the immediate postoperative period. In contrast, intravenous administration of the

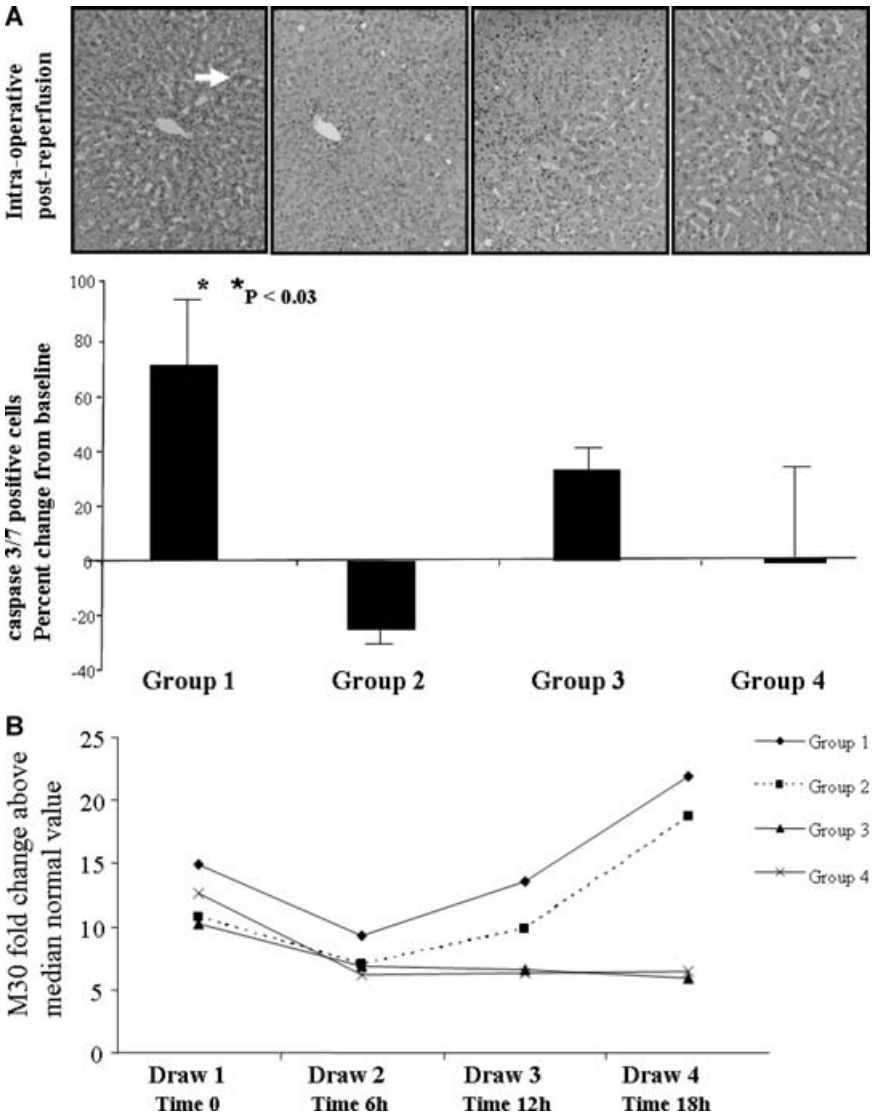

Figure 1: Apoptosis is reduced in human livers treated with IDN-6556 following liver transplantation. (A) Top panel: Representative photomicrographs of intraoperative, postreperfusion liver specimens stained for caspase 3/7 are depicted (see 'Methods'). Note red granules of caspase 3/7 immunoreactivity within the hepatic tissue (white arrow in the first panel). Lower panel: Liver biopsies obtained from donor back-table preperfusion livers and intraoperative postreperfusion livers were analyzed for caspase 3/7 activity ($n = 99$). The donor back-table preperfusion liver data was considered baseline. There was a significant percent increase change from baseline of caspase 3/7 positive cells/hpf in Group 1, but not in Groups 2, 3 and 4 ($p < 0.03$). (B) Serum samples collected from liver transplant recipients prior to intravenous administration of IDN-6556, but following reperfusion, were analyzed for apoptosis via the M30-Apoptosense ELISA assay (marker for CK18Asp396 ('M30') neoepitope). Eighteen hours after reperfusion, Group 1 (placebo) showed higher concentrations of M30 when compared to Groups 3 and 4 ($p < 0.0001$ as compared to Group 3, $p < 0.001$ as compared to Group 4) ($n = 99$) (Normal 'M30'value: median = 121.5 U/L).

drug was associated with neutrophil accumulation in the allograft.

Is treatment with IDN-6556 well tolerated?

Table 4 summarizes adverse events occurring during the first 30 days after transplant. Note that Group 2 experienced fewer adverse events overall in the immediate postoperative period, and did not have an allograft experience delayed graft function or primary non-function (PNF) as compared to other treatment groups. Deaths occurred in all treatment groups. Group1: two deaths, one from myocardial infarction and the other from PNF of the transplanted graft; Group 2: one death from pulmonary hypertension; Group 3: one death from PNF; Group 4: one death from sepsis. There was no significant difference between Groups 1, 2 and 3 as it related to intensive care unit stay or overall hospital stay (data available from Mayo Clinic, Rochester only; $n = 35$): Group 1: intensive care unit stay (1.9 ± 0.7 days), overall hospital stay (12.1 ± 7.6 days); Group 2 (1.1 ± 0.3 days and 10.1 ± 5.1 days); Group 3 (1.2 ± 0.4 and 11.9 ± 4.2 days). In contrast, Group 4 had comparable intensive care unit days but a higher average of overall hospital days (2.1 ± 1.8 and 20.7 ± 19.1 days) secondary to one patient with delayed graft function. These data support the conclusion that treatment with IDN-6556 in the flush and storage solutions during liver transplantation is well tolerated.

Discussion

The principal findings of this study relate to the effects of the pan-caspase inhibitor, IDN-6556 in human liver transplantation. These results suggest that treatment with IDN-6556 added to the storage and flush solutions: (1) reduces immediate reperfusion-mediated apoptosis; (2) ameliorates postoperative liver injury and (3) and appears to be well tolerated. Interestingly, when included in the

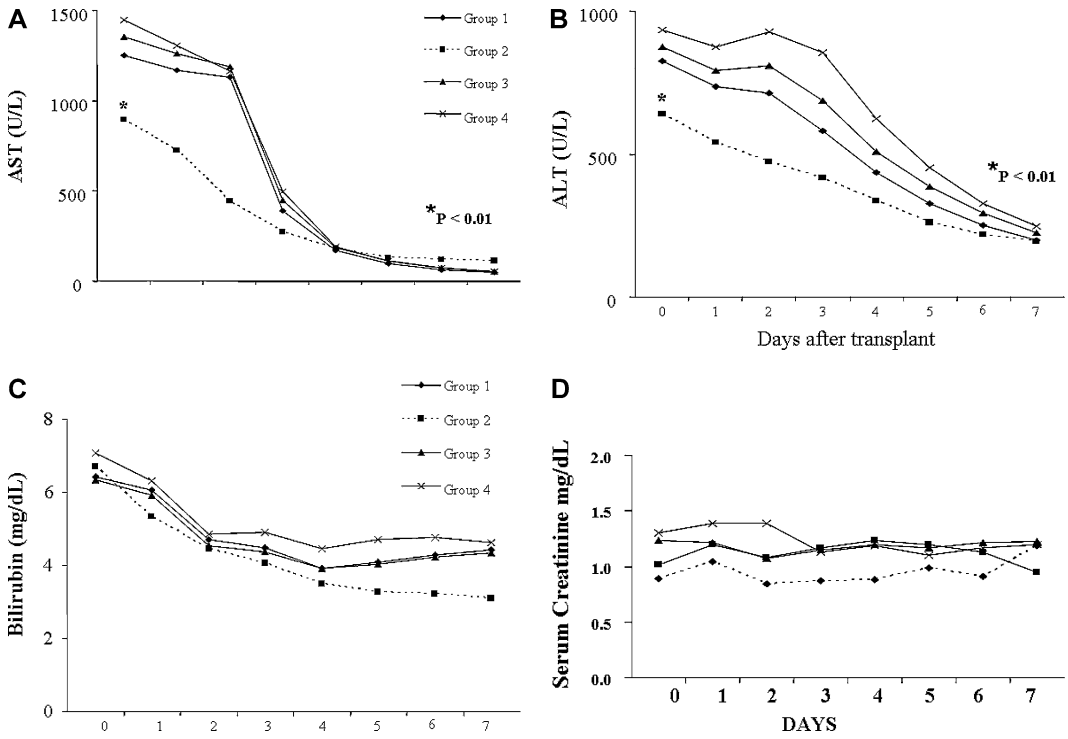

Figure 2: Liver injury is reduced by IDN-6556 treatment of liver allografts. (A & B) Serum AST and ALT obtained from liver transplant recipients daily for the first 7 days postoperatively (n = 99). Note that all values were obtained after administration of the drug which was given at the time of hepatic artery anastimosis, prior to the subsequent determination of the AST/ALT values obtained after the patient reached the intensive care unit. Group 2 showed a significant reduction in serum AST/ALT levels postoperatively when compared to Group 1 (*$p < 0.01$ for AST, *$p < 0.01$ for ALT). (C & D) Serum creatinine and total bilirubin levels demonstrated no significant difference between groups.

storage and flush solution plus administered intravenously for 24 h after liver transplantation, these salutary effects were overturned, perhaps due to accumulation of neutrophils within the graft.

To assess apoptosis we utilized the TUNEL assay, caspase 3/7 activity and the M30 Apoptosense ELISA, which quantitated the apoptosis-associated CK18Asp396 ('M30') neo-epitope in serum samples from liver transplant recipients. The M30 antibody recognizes a neo-epitope, exposed after caspase-mediated cleavage of CK18 after the aspartic acid residue 396 (CK18Asp396) (18). Cleavage at this position occurs early during apoptosis and is mediated by caspase-9, as well as caspase-3 and -7 (19). The increase of CK18Asp396 observed during apoptosis is blocked by caspase inhibition (20). The remarkably elevated serum levels of this apoptosis biomarker suggest that caspase-dependent, cellular apoptosis, is a prominent feature of liver transplantation surgery. How much of the biomarker originates from the transplanted organ and tissues remain unclear. However, the observation that M30 serum levels were several-fold higher than normal levels in all treatment arms, despite administration of a pan-caspase inhibitor, suggests other tissues have an abundant apoptosis response to the complex processes involved in liver transplantation. Nonetheless, the consistent reduction in M30 concentrations during the first 24 h after transplantation from serum samples of patients given IDN-6556 intravenously, implicates the liver as a substantial source of circulating biomarker. In analyses of liver cell-specific apoptosis, by day 7, liver specimens obtained from Group 2 (IDN-6556 in perfusate alone) showed a marked reduction in apoptosis as depicted by TUNEL assay and caspase 3/7 positive cells/hpf. These observations demonstrate that treatment with IDN-6556 in the cold storage and flush solutions only may reduce human liver allograft apoptosis after CI/WR injury.

An unanticipated observation occurred in this study as intravenous administration of IDN-6556 abrogated the beneficial effect of organ alone exposure. Indeed, the result was dose dependent, as the higher overall concentration of the drug sensed by the transplant recipient, the less favorable it was. Group 4 received the highest concentration of drug and its effect on reduction of apoptosis, postoperative liver injury, allograft rejection and inflammation was minimal to none. The mechanism for this phenomenon is unknown. It is possible that the physiologic turnover of neutrophils, especially those infiltrating the liver (10) when higher doses of IDN-6556 is administered, may account

Table 3: Neutrophil accumulation in liver allografts 7 days postliver transplantation

Histologic review of biopsies on day 7 (Mayo Clinic, Rochester)

		Neutrophil grade[1]			
Study group	N	1+	2+	3+	Zone of distribution[2]
1	6	60%	20%	20%	1
2	6	100%	0%	0%	Nonzonal
3	8	85.7%	14.3%	0%	Nonzonal
4	8	71.4%	14.3%	14.3%	1

[1]Grade of neutrophil disbursement corresponding to increasing neutrophil count per high power field (40×).
[2]Distribution of neutrophils in liver parenchyma (Zones 1, 2, 3 or non-zonal).

Table 4: Adverse events occurring in the course of the study

	Postoperative adverse events[1]			
	Group 1[2] n = 23	Group 2[2] n = 23	Group 3[2] n = 27	Group 4[2] n = 26
Hepatic artery stenosis	1	0	1	1
Portal vein thrombosis	1	0	2	0
Biliary stenosis	2	1	3	2
Blood dyscracias[3]	4	7	7	4
Neurologic changes[4]	8	5	8	9
Cardiac events[5]	6	2	3	8
Renal insufficiency[6]	4	3	6	4
Delayed graft function	2	0	2	2
Primary nonfunction	1	0	1	0
Re-transplantation	0	0	0	0
Death	2	1	1	1

[1]Adverse events occurring within 30 days after liver transplantation.
[2]Number of occurrences of events (one patient may have experienced multiple events).
[3]Anemia.
[4]Depression, anxiety, confusion/agitation, double vision, seizures, loss of short-term memory.
[5]Atrial fibrillation, congestive heart failure, pericardial effusion, bradycardia, supraventicular tachycardia, bundle branch block, myocardial function (not ending in death).
[6]Renal insufficiency as described as serum creatinine ≥2.0, no renal replacement necessary, resolved by 4-month evaluation.

for this paradoxical phenomenon. Apoptosis is responsible for neutrophil turnover and is considered essential for the resolution of the inflammatory process (23). Caspases are major executors of the apoptotic program in human neutrophils (24). Agents that can inhibit caspase activation, can thereby hinder neutrophil apoptosis and, in doing so, may prolong and/or worsen an inflammatory response (24–26) and potentially result in an overall net negative effect. In this respect, administration of a caspase inhibitor in the later phases of CI/WR injury could have a detrimental consequence. Our data demonstrating enhanced neutrophil infiltration in the liver of individuals receiving postoperative intravenous IDN-6556 supports this interpretation.

Besides caspases, additional proteases have been implicated in CI/WR injury including calpains and cathepsin B (27,28). Caspase inhibitors have been reported to inhibit cathepsins at high dose (29). Therefore, it is possible that the agent used in this study IDN-6556 inhibits other proteases in addition to caspases accounting for its salutary effects.

In the IDN-6556 Phase I clinical trial to evaluate safety (15), intravenous administration (0.1–1.0 mg/kg) four times daily for 7 days in patients with mild liver impairment was proven to be safe. Adverse events were minimal; the main one being inflammation and/or phlebitis at the infusion site. This latter study also observed a beneficial effect; clinically and statistically significant reduction in serum ALT/AST levels, which rapidly returned to pretreatment levels after discontinuation of the drug. This study population differs greatly from the population investigated in the current phase II clinical trial (Phase I: mild hepatic impairment; Phase II: end-stage liver disease requiring transplant). Local anti-apoptotic therapy may be prudent for the liver transplant population. In fact, it has been shown that systemic inhibition is not necessary for IDN-6556 to afford liver protection from CI/WR injury. In animal studies, (13), IDN-6556 added to the UW solution alone was the most effective at inhibiting CI/WR-induced apoptosis compared to other caspase inhibitors and systemic administration provided no extra benefit. In this study we have shown IDN-6556 most efficacious in Group 2 (treatment to organ alone), with precipitous drop of AST/ALT immediately postoperatively, no increase in hospital stay, no occurrences of delayed graft function, and no PNF or retransplantation. Given these promising but limited observations, larger studies are justified to confirm these provocative findings.

In summary, CI/WR-induced hepatic apoptosis contributes to the development and exacerbation of PNF and delayed graft dysfunction (30) and a higher incidence of both acute and chronic rejection (1). Therefore, inhibition of apoptosis is a strategy to improve outcomes after liver transplantation. The pan caspase inhibitor, IDN-6556 when administered in cold storage and flush solutions during liver transplantation may provide local therapeutic protection against CI/WR-mediated apoptosis and injury. This study was conducted using a limited number of patients at centers where the incidence of PNF was minimal. Whether this therapeutic approach will be sufficient to decrease allograft dysfunction or permit safe clinical utilization in current extended criteria donors will require further study.

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Appendix

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