Characterization of the Caspase Inhibitor IDN-1965 in a Model of Apoptosis-Associated Liver Injury

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ABSTRACT

Previous studies have shown that caspase inhibitors are effective at protecting against anti-Fas antibody (α-Fas)-mediated liver injury/lethality. The purpose of these experiments was to characterize more fully the efficacy of a broad-spectrum, irreversible caspase inhibitor, IDN-1965 ([1,3-dimethylindole-2-carbonyl]valinyl)-3-amino-4-oxo-5-fluoropentanoic acid), in this model and the role of caspase inhibition in long-term protection. The ED₅₀ for IDN-1965 by i.p. administration, based on alanine aminotransferase activities, was 0.14 mg/kg. The caspase inhibitor was also efficacious when administered intravenously and orally (ED₅₀ values of 0.04 and 1.2 mg/kg, respectively). Histologically, marked reduction in Fas-induced apoptosis with IDN-1965 (1 mg/kg, i.p.) was apparent at 6 h. Also, caspase 3-like activities were decreased in a dose-dependent manner, but the inhibition of caspase activity was transient. Immunohistochemical studies demonstrated that IDN-1965 greatly reduced the activation of caspase 3. In survival studies, a single i.p. treatment of 1 mg/kg IDN-1965 or continuous i.p. infusion via osmotic pumps completely blocked lethality measured up to 7 days after α-Fas administration. IDN-1965 was also effective in inhibiting liver injury when administered as long as 3 h after or 1 h before α-Fas administration. Lastly, Western blot analysis demonstrated that processing of caspases 3, 6, and 8, as well as Bid (a protein responsible for the release of mitochondrial cytochrome C and amplification of the apoptotic cascade) was inhibited by IDN-1965. In conclusion, the broad-spectrum caspase inhibitor IDN-1965 is markedly effective at inhibiting Fas-mediated apoptosis by multiple routes of administration. The therapeutic potential of caspase inhibitors appears promising for the treatment of apoptosis-mediated liver injury based on potency and postinsult efficacy.

Programmed cell death, or apoptosis, can be initiated by the activation of the CD95 (APO-1/Fas) receptor, as well as the closely related tumor necrosis factor receptor 1 (Galle and Krammer, 1998; Nagata, 1999). These receptors are located in relatively high concentrations in the liver (Watanabe-Fukunaga et al., 1992). Normally, apoptosis is thought to control the number of hepatocytes within the liver (Benedetti et al., 1988). However, under pathological conditions, uncontrolled cell death leads to tissue injury or even organ destruction. Abnormally high amounts of apoptosis occur clinically in a number of liver diseases, including alcoholic hepatitis, hepatitis B or C viral infection, and Wilson’s disease, as well as in livers of patients undergoing orthotopic liver transplantation (Kerr et al., 1979; Kawahara et al., 1994; Strand et al., 1998; Tannapfel et al., 1999). Moreover, the CD95 pathway has been implicated in all these conditions (Galle et al., 1995; Strand et al., 1998; Taieb et al., 1998; Tannapfel et al., 1999).

Experimentally, the apoptotic pathway in hepatocytes can be initiated by activation of the CD95 pathway by administration of an anti-Fas antibody (α-Fas), which is agonistic for the Fas receptor (Ogasawara et al., 1993). Administration of an α-Fas antibody results in marked hepatocellular apoptosis beginning as early as 2 h after administration, with the onset of fulminant hepatitis and lethality occurring near 5 h. Therefore, this model is a valuable system to study Fas-mediated hepatocellular apoptosis. Apoptosis triggered by the Fas pathway occurs, at least in part, by the activation of intracellular enzymes called caspases. Caspases are a family of cysteine proteases that are normally found in an inactive pro-form (Cohen, 1997). Upon activation of the apoptotic pathway, initiator caspases (i.e., caspases 8 and 9) are converted to their active forms, which in turn activate downstream caspases (i.e., caspases 3, 6, and 7). Cellular targets of the effector caspases include endonucleases and cytoskeletal proteins (for review, see Earnshaw et al., 1999). Additionally, Bid, a protein responsible for the release of mitochondrial cytochrome C and amplification of the apoptotic cascade, is also activated by caspases (Luo et al., 1998). Ultimately, this

ABBREVIATIONS: α-Fas, anti-Fas antibody; Bid, protein responsible for the release of mitochondrial cytochrome C and amplification of the apoptotic cascade; IDN-1965, N-[(1,3-dimethylindole-2-carbonyl]valinyl]-3-amino-4-oxo-5-fluoropentanoic acid; DMSO, dimethyl sulfoxide; ALT, alanine aminotransferase; DTT, dithiothreitol; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; PBST, phosphate-buffered saline/0.1% Tween 20; DEVD-amc, aspartyl-glutamyl-valinyl-aspartyl-aminomethylcoumarin.
pathway ensures the self-destruction of the cell with the characteristic morphological features of apoptosis, including nuclear fragmentation, cellular shrinkage, and acidophilic staining of the cytoplasm.

Activation of caspase has been demonstrated in the α-Fas model, and previous studies have shown that caspase inhibitors [carbobenzyloxy-valinyl-alanyl-aspartyl]-fluoromethylketone (Z-VAD-FMK), tyrosinyl-valinyl-alanyl-aspartyl-chloromethylketone (YVAD-CMK), and asparaginyl-glutamyl-valinyl-asparaginyl-aldehyde (DEVD-CHO)] are effective in eliminating Fas-mediated liver injury (Rodriguez et al., 1996; Rouquet et al., 1996; Kunstle et al., 1997; Suzuki, 1998). However, no reports have thoroughly characterized a caspase inhibitor for efficacy and its effects on caspase activation. The aim of this paper is to more fully characterize the caspase pathway in the liver and to discuss for indications where abnormally high amounts of apoptosis occur.

**Experimental Procedures**

**Materials**

α-Fas was purchased from PharMingen (Jo-2; PharMingen, San Diego, CA). Pentobarbital (Nembutal) was purchased from Western Medical Supply (Arcadia, CA). Alzet osmotic pumps were purchased from Alza Corp. (Palo Alto, CA). IDN-1965 was synthesized as described (U.S. patent no. 5,869,519). All other materials were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

**Animals and Treatment**

Male BALB/c mice (Harlan Sprague-Dawley, Indianapolis, IN) weighing approximately 20 to 25 g were provided food and water ad libitum before the experiment. Animals were acclimated for at least 7 days before experimentation.

α-Fas Model. In all studies, α-Fas was injected i.v. at a dose of 200 μg/kg via the tail vein. IDN-1965 was administered in either saline, DMSO:saline (1:1 ratio), or DMSO:water (1:1) at times indicated in the figures. Controls were administered similar volumes of appropriate vehicle (4 ml/kg). Preliminary experiments determined that different vehicles alone do not affect α-Fas-mediated liver injury in this model (data not shown). When comparing efficacy by multiple routes of administration (Table 1), IDN-1965 was administered i.p., s.c., or i.v. in sterile saline immediately after administration of α-Fas at concentrations indicated in Table 1. In osmotic pump studies, isolurane-anesthetized mice received Alzet osmotic pumps implanted into the peritoneum and were allowed to recover for 24 h. Pumps contained either 50% water:DMSO or IDN-1965 (30 mg/ml) in 50% water:DMSO. In most experiments, animals were euthanized with pentobarbital (50 mg/kg, i.p.) 1.5 to 24 h after the administration of antibody. In lethality studies, surviving mice were allowed to live for 7 days after α-Fas administration before euthanization with pentobarbital. Plasma was harvested for measurement of alanine aminotransferase (ALT) activities. Portions of liver were fixed in formalin (10% in neutral-buffered saline) for immunohistochemical and morphological analyses, as well as frozen in liquid nitrogen for caspase activity determination.

**Assays**

**Caspase Activity.** Livers were frozen in liquid nitrogen immediately upon collection and stored until processed. Tissues were homogenized in ice-cold hypotonic buffer (10 mM HEPES, pH 7.4; 42 mM KCl; 50 mM MgCl₂·6H₂O) containing 1 mM dithiothreitol (DTT), 0.5% (w/v) CHAPS (Sigma Ultrapure), and a cocktail of protease inhibitors [100 μM EGTA, 100 μM EDTA, 1 μM µg/ml aprotinin, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and centrifuged at 12,000g for 15 min at 4°C. Protein concentrations of the resulting supernatant suspension were determined routinely using a bicinchoninic acid method (Pierce, Inc., Rockford, IL) with bovine serum albumin as the standard. The tissue sample extracts were assayed for caspase enzyme activity by monitoring the fluorescent product of the cleavage of DEVD-amc. Briefly, 200 μg of protein/well was added to 96-well microtiter plates and diluted to 50 μl by addition of ice-cold buffer (consisting of 20 mM HEPES [pH 7.5], 1 mM EDTA, 10% sucrose, and 0.1% CHAPS) to which DTT was added fresh before the start of every assay at a final concentration of 10 mM. The enzyme assay was initiated by addition of the DEVD-amc substrate to a final concentration of 12.7 μM (100 μl of a 20 μM stock). The substrate cleavage activity was measured at time 0 and every 30 min thereafter for 2 h in a fluorescent plate reader (excitation wavelength: 360/40 nm; emission wavelength: 460/40 nm).

**Immunohistochemical Analysis of Activated Caspase 3.** CM-1 antibody, which recognizes the p18 subunit of the cleaved caspase 3 (activated caspase 3), was generated in rabbits, purified, and characterized as previously described (Srinivasan et al., 1998b). Formalin-fixed, paraffin-embedded livers were cut into 5-μm sections and rehydrated by routine methods. After elimination of endogenous peroxidase activity with 3% H₂O₂, tissues were subjected to an antigen retrieval step (boiling in 0.1 M sodium citrate for 15 min), followed by incubation in blocking buffer for 1 h. Tissue sections were then exposed to 0.4 μg/ml antibody (CM-1) for 1 h in blocking buffer. After extensive washing, amplification of the signal was performed using a biotinylated antibody kit (Vector Laboratories, Burlingame, CA) with diaminobenzidine as the indicator. Tissues were counterstained with Mayer’s hematoxylin. Negative controls consisted both of tissues incubated without primary antibody or with nonspecific IgG antibody.

**Western Immunoblotting.** Frozen liver tissues from control and α-Fas-treated mice were thawed in lysis buffer (50 mM KCl, 50 mM Pipes [pH 7.4], 10 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 0.1 μM PMSF, 1 mM cytochalasin B, 2 μM µg/ml leupeptin, 1 μg/ml pepstatin A, and 10 mg/ml aprotinin) and homogenized on ice using a Polytron tissue homogenizer. Ground tissue was then further homogenized using a Dounce homogenizer (100 strokes), transferred to 1.5-ml microfuge tubes, and subjected to five rounds of dry ice/70°C freeze-thaw cycles. Samples were centrifuged at 10,000g at 4°C for 20 min to remove solid material. Supernatants were centrifuged for 1 h at 100,000g at 4°C. Protein concentrations of the S100 supernatants were measured using the Protein Assay Kit II (Bio-Rad, Hercules, CA) with bovine serum albumin as the calibration standard. Western blotting of the S100 supernatants was done as previously described (Krebs et al. 1999). Briefly, liver extracts (60 μg of protein per lane)

**TABLE 1**

Potency of IDN-1965 by multiple routes of administration in the α-Fas model of liver injury

<table>
<thead>
<tr>
<th>Route of Administration</th>
<th>ED₅₀ (μg/kg)</th>
<th>95% Confidence Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.p.</td>
<td>0.14</td>
<td>0.06–0.34</td>
</tr>
<tr>
<td>i.v.</td>
<td>0.04</td>
<td>0.02–0.09</td>
</tr>
<tr>
<td>p.o.</td>
<td>1.25</td>
<td>0.46–3.52</td>
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were resolved by sodium dodecyl sulfate-polyacrylamide gel elec-
trophoresis on 12% or 16% gels (Novex, La Jolla, CA) and trans-ferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford,
MA). Membranes were blocked in phosphate-buffered saline/0.1%
Tween 20 (PBST) + 0.4% casein (I-block, Tropix, Bedford, MA). Blots
were incubated in 1 μg/ml primary antibody diluted in PBST/casein
for 1 h. Following three washes in PBST, blots were incubated for 1 h
in 1:15,000 dilutions of horseradish peroxidase-conjugated goat anti-
rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove,
PA) in PBST/casein. Following the secondary antibody incubations,
the blots were developed using the ECL-plus kit (Amersham Phar-
macia Biotech, Piscataway, NJ). The blots were visualized using the
STORM fluorescence imager (Molecular Dynamics, Sunnyvale, CA).
The antibodies used were against caspase 8 (Srinivasan et al.,
1998a), caspase 3 (Srinivasan et al., 1998b), caspase 3 large subunit
(Srinivasan et al., 1998a), human Bid (Ref., kind gift of Dr. X. Wang,
University of Texas Southwestern Health Science Center, Dallas,
TX), and caspase 6 large subunit (C6LS-1, Idun Pharmaceuticals).
The rabbit polyclonal antibody to the large subunit of caspase 6 was
generated using a protocol identical to that used to generate CM-1
(Srinivasan et al., 1998b), except in the peptide sequence of the
immunogen. The sequence of the peptide used to generate the rabbit
sera was NH2-CRGNQHDPVPLDVPDOH-COOH conjugated to key-
hole limpet hemocyanin. The sequence corresponds to amino acids
163 through 179 of human caspase 6. All antibodies used were
affinity-purified rabbit polyclonals, except the antibody to human
Bid, which was used as an unpurified rabbit serum.

Morphological Analysis. Liver sections (5 μm) were stained
with H&E for morphological analysis. Representative photomicro-
graphs are shown in the figures.

ALT Activities. ALT activities were determined using a standard
diagnostic kit (Sigma).

Statistical Analyses. Data are expressed as the mean ± S.E.M.
ED50 dose-response curves were calculated by plotting ALT as a
percentage of α-Fas-treated positive controls-vehicle controls, fol-
lowed by nonlinear regression analysis using GraphPad Prism Soft-
ware, Inc. (San Diego, CA). When comparing significant differences
between groups, either a one-way or two-way analysis of variance
was performed followed by either a Student Newman-Keuls test or
Bonferroni’s post hoc test. Differences were considered significant if
p < 0.05.

Results

Previous studies demonstrated that administration of
α-Fas antibody results in massive hepatocellular apoptosis and peak
ALT activities near 6 h postadministration (Ogas-
sawara et al., 1993). When administered simultaneously with
α-Fas (200 μg/kg, i.v.), i.p. administration of IDN-1965
dose dependently decreased in ALT activities when mea-
sured 6 h after α-Fas administration. A representative ex-
periment is shown in Fig. 1, and the average ED50 based on
three experiments for IDN-1965 was 0.14 ± 0.08 mg/kg. To
determine the potency of IDN-1965 by multiple routes of
administration, the ED50 for liver injury after i.p., p.o., and
i.v. administration was determined (Table 1). The potency
was similar for i.p. and i.v. administration, but p.o. admin-
istration was less potent. Histologically, massive hepatocel-
lar apoptosis and hemorrhaging was noted in livers treated
with α-Fas after 6 h, whereas only a few hepatocytes were
apoptotic in livers treated with α-Fas and IDN-1965 at doses of
1 mg/kg or higher (Fig. 2, A and B).

To investigate the effect of IDN-1965 on caspase activation,
caspase 3-like activities were measured in the liver between
1.5 and 6 h after α-Fas administration. Caspase activities
markedly increased 3 h after α-Fas administration and re-
mained elevated over the 6-h period (Fig. 3A). Coadministra-
tion of 3 mg/kg IDN-1965 by i.p. injection inhibited caspase
activities at 3 h, but caspase activity increased to similar
levels as the α-Fas-treated livers by 6 h. In contrast, a single
i.p. injection of 10 mg/kg IDN-1965 completely inhibited
caspase activities over the 6-h period (Fig. 3A). ALT activities
at 6 h were inhibited by 93 and 98% in the 3 and 10 mg/kg
dose groups, respectively (Fig. 3B).

An immunohistochemical analysis of livers for activated
caspase 3, using an antibody (CM-1) that recognizes the
processed (active) form of caspase 3 in the liver of α-Fas-
treated mice also revealed a similar time course of caspase
activation. Positive staining occurred in a few hepatocytes
1.5 h after α-Fas administration, but was pronounced in a
number of hepatocytes by 3.0 h (Fig. 4, A and B). Detection of
activated caspase 3 protein was extensive by 4.5 h post-α-Fas
administration (Fig. 4C) and remained high over the 6-h
period (not shown). These data coincide with caspase activi-
ties measured by DEVD-amin cleavage (Fig. 3B). When α-Fas-
treated mice were cotreated with 10 mg/kg IDN-1965, few
CM-1 positive cells were noted even 6.0 h after α-Fas injec-
tion (Fig. 4D). Negative controls (using nonspecific IgG in-
stead of CM-1 antibody) had little or no staining, indicating
the specificity for the antibody (not shown).

Increases in caspase activation 6 h after α-Fas administra-
despite cotreatment of IDN-1965, raised the question whether liver
injury would occur at later time points. Therefore, the effect of a single dose of IDN-1965 (1 mg/kg) on
survival and liver injury in α-Fas-treated mice was assessed
over a 7-day period. This was also compared with a continu-
sous administration of IDN-1965 via an osmotic minipump
implanted i.p. over the same period of time. In this experi-
ment, 7 of 10 α-Fas-treated mice died over a 7-day period
after α-Fas administration, with the majority of mice dying
between 4 and 12 h post-treatment. ALT activities 6 h after
α-Fas administration in the surviving mice (n = 3) were
6215 ± 939 IU/l (Fig. 5) and decreased to 1364 ± 542 IU/l by
24 h. In contrast, a single dose of 1 mg/kg IDN-1965 com-
pletely eliminated $\alpha$-Fas-induced lethality up to 7 days post-treatment and markedly lowered ALT activities at 6 h (152 ± 140 IU/l). However, ALT activities were not significantly different than $\alpha$-Fas-treated mice at 24 h (969 ± 251 IU/l). By 48 h, ALT activities of the $\alpha$-Fas-treated mice decreased to control levels. Administration of IDN-1965 via an osmotic minipump (0.36 mg/kg/h) 24 h before $\alpha$-Fas injection significantly blocked liver injury at both the 6 and 24 h time points (Fig. 5). $\alpha$-Fas-induced lethality was also completely blocked by constant infusion, compared with mice receiving vehicle from the osmotic pump ($n = 6$).

Histologically, this time course study confirmed that $\alpha$-Fas-treated livers contained numerous apoptotic hepatocytes at 6 h, which progressed to massive areas of necrosis by 24 h. By 7 days, fibrotic areas were present with inflammatory infiltrates and numerous mitotic cells, indicating hepatocellular regeneration (Fig. 6A). In contrast, a single dose of 1 mg/kg IDN-1965 blocked apoptosis in most hepatocytes at 6 h, but numerous apoptotic, as well as necrotic, areas were evident by 24 h. By 7 days, livers had areas of fibrosis with mild inflammation and mitotic hepatocytes, but the extent was much less than that of $\alpha$-Fas treatment alone at all time points (data not shown). Lastly, livers were essentially normal in $\alpha$-Fas-treated mice coadministered IDN-1965 via the osmotic pump 7 days after administration, with few apoptotic hepatocytes at the 6 h time point (Fig. 6B). Livers from control mice receiving either IDN-1965 or vehicle via osmotic mini-pumps were unremarkable.

To determine a window of efficacy of IDN-1965 in this model, the caspase inhibitor was administered both several hours before and after $\alpha$-Fas administration. In predosing experiments, IDN-1965 significantly protected the liver from $\alpha$-Fas when administered up to 1 h before $\alpha$-Fas administration (Fig. 7A). Treatment with 3 mg/kg IDN-1965 3 or 5 h
before α-Fas resulted in no protection of the liver. To determine how effective IDN-1965 is when administered after the α-Fas insult, mice were treated with IDN-1965 0 to 4 h after α-Fas. As shown in Fig. 7B, IDN-1965 significantly inhibited α-Fas-induced liver injury when administered as late as 3 h post-α-Fas administration. Administration of IDN-1965 3 h post-α-Fas still reduced liver injury by 61%, but administration 4 h after was not efficacious.

To determine whether IDN-1965 alters caspase processing, Western blot analysis was used to examine the effect of IDN-1965 on α-Fas-induced processing of caspases 8, 3, and 6, 3 h after α-Fas administration. The effect of IDN-1965 cotreatment on Bid cleavage was also determined. α-Fas administration resulted in the disappearance of pro-caspase 8, which was inhibited by IDN-1965 (Fig. 8A). Additionally, the processed forms of caspases 3 and 6 present after α-Fas administration were not detected when IDN-1965 was coadministered with α-Fas (Fig. 8, B and C). The activated form of caspase 3 was detected only in livers of α-Fas-treated mice, but not in mice cotreated with IDN-1965 (Fig. 8D). Lastly, Bid, a substrate for various caspases, was cleaved in livers of α-Fas-treated mice, but not in livers of mice cotreated with IDN-1965.

Discussion

Excess apoptosis, which appears to be a detrimental process in a number of liver diseases, may be effectively treated by inhibition of caspases. Liver diseases where abnormally large amounts of apoptosis occur include Wilson’s disease, allograft rejection, and both viral and alcoholic hepatitis (Kerr et al., 1979; Kawahara et al., 1994; Strand et al., 1998; Tannapfel et al., 1999). Moreover, CD95/Fas-mediated apoptosis has been associated with these diseases. For example, increased CD95 expression was seen in hepatocytes from patients with alcoholic liver disease, as well as in livers of patients with acute liver failure diagnosed with Wilson’s disease (Galle et al., 1995; Strand et al., 1998; Taieb et al., 1998; Tannapfel et al., 1999). The beneficial effects of caspase inhibitors in models of apoptotic liver injury are now well established, but reports have not fully characterized a caspase inhibitor in these systems. Therefore, the major ob-
Objective of this study was to more fully evaluate a caspase inhibitor in the α-Fas model of liver injury and lethality where apoptosis via the FAS/APO1/CD95 system predominates. Additionally, the effect of this drug on the activation of a number of caspases and Bid within the liver was also investigated.

IDN-1965 is an irreversible caspase inhibitor with potent activity on a number of caspases. For example, the dissociation constant of enzyme inhibitor complex (K_i) for IDN-1965 is less than 0.08 and 0.03 μM for the apical caspases 8 and 9, respectively (Wu and Fritz, 1999). Based on the nature of the caspase cascade, inhibition of these apical caspases may be critical for the apoptotic cascade to be halted. Caspase 8 associates with FADD/MORT1 adaptor and is the first caspase activated in the Fas pathway (Fernandes-Alnemri et al., 1996). Self-activation of caspase 8 leads to the direct activation of caspase 3 and cleavage of Bid (Li et al., 1998). Cleaved Bid induces cytochrome c release from the mitochondria which in turn activates caspase 9 in the presence of apaf-1 (Li et al., 1997). Caspase 9 then can also activate caspase 3 directly and thus the cascade amplifies for destruction of the cell. Because of the potency and effectiveness of IDN-1965 to inhibit the apical caspases, efficacy was predicted in this model when administered simultaneously with α-Fas. This was evidenced by ED_50 values of approximately 0.1 to 0.3 mg/kg after i.p. and i.v. routes of administration, respectively. Orally administered IDN-1965 was also effective, but at an order of magnitude lower than i.p. administration, possibly due to the lower bioavailability of the drug.

Since IDN-1965 also has a relatively low K_i for caspase 3 (0.96 μM) and caspase 6 (0.04 μM; Wu and Fritz, 1999), the drug may also have the potential to inhibit apoptosis after activation of the apical caspases has occurred. This was ap-
binding to caspase 3 is 0.78 min$^{-1}$, resulting in a high rate of inactivation (Wu and Fritz, 1999). Therefore, the high activity toward caspase 3 may also explain the effectiveness of IDN-1965 in this model, further supporting the hypothesis that broad-spectrum caspase inhibition may be necessary for effective therapy due to the amplifying nature of the caspase cascade. Additionally, recent data suggest that caspase 3 can activate the upstream caspases 8 and 9, as well as the BH3 domain containing protein Bid, suggesting caspase 3 can further amplify the caspase cascade (Woo et al., 1999). Inhibition of caspase 3 activity may be a crucial element, but not the only one, to block further programmed cell death. Therefore, although caspase 3 inhibition is important, caspase 3$^{-/-}$ mice were still susceptible to α-Fas-mediated liver injury and lethality, although the α-Fas-mediated effects were delayed (Woo et al., 1999).

Despite its irreversible nature, the effect of IDN-1965 on inhibition of α-Fas-induced caspase activity in the liver appears to be short-lived. This is supported by the observation that administration of 1 or 3 mg/kg IDN-1965 significantly inhibited liver caspase 3-like activation 3 h after α-Fas administration but by 4.5 h, caspase activation was equivalent to those of α-Fas-treated mice (despite near complete inhibition of liver injury at 6 h). A number of possibilities can explain this finding, including a relatively rapid clearance of the drug. Preliminary studies have determined plasma half-life in rats after i.v. administration for IDN-1965 is 40 min (data not shown). The predose study further supports this hypothesis since no efficacy was demonstrated if drug was administered 3 h before α-Fas. Transient inhibition may also be due to ongoing expression (de novo synthesis) of one or a number of caspases, ultimately resulting in active caspase, but these studies have not been reported. There may be other protective effects of IDN-1965, but activity of IDN-1965 against other serine/cysteine proteases is low. The $K_i$ of IDN-1965 against cathepsin B, calpain I, and calpain II are 1360, 24.3, and 11.6 μM, respectively (Joe Wu, unpublished data). Regardless of the mechanism(s), caspase activity was initially inhibited by IDN-1965 at lower doses, and either multiple or continuous dosing of a caspase inhibitor may be necessary for optimal long-term efficacy.

The increases in caspase activity without a significant rise in ALT activity at the 6 h time point raised the question whether α-Fas-induced injury continues to occur after the 6 h time point, despite treatment with IDN-1965. This was addressed by comparing Fas-mediated liver injury after a single dose of IDN-1965 to continuous administration of the drug. Indeed, although there was little evidence of α-Fas-induced liver injury at 6 h with coadministration of a single dose of IDN-1965, ALT activities at 24 h were similar to controls. Additionally, at this time point, both apoptosis and necrosis were evident in both treatment groups. Therefore, these data suggest that the increase in caspase activity initially inhibited by IDN-1965 will eventually lead to liver apoptosis at later time points. Continuous administration of IDN-1965 via an osmotic minipump (a delivery equaling 0.36 mg/kg/h), however, was extremely effective in reducing liver injury, as evidenced by both ALT activities and histological evaluation throughout the 7-day period. It appears therefore that as long as adequate concentrations of inhibitor are achieved, the caspase cascade and ultimately apoptosis in this model will be inhibited.
Another objective of this study was to determine which caspases in the liver become activated after in vivo α-Fas administration and how IDN-1965 affected their activation. In particular, the upstream caspase 8, as well as the downstream caspases 3 and 6 were examined. Consistent with previous reports demonstrating the processing of pro-caspase 8 and pro-caspase 3 after α-Fas administration (Yin et al., 1999), the cleaved form of caspase 3 using the CM-1 antibody was also clearly detected. In contrast, processing of caspase 6 was also detected, a finding contrary to Woo et al. (1999). The reason for the discrepancy is not known, but could be due to the use of different primary antibodies and methodology. These studies also indicated that IDN-1965 inhibited the processing of all three caspases. Finally, the effect of caspase inhibitors on the processing of Bid was investigated. Bid is activated by caspase 8 and is an essential component of the caspase cascade, responsible for the release of cytochrome c from the mitochondria (Li et al., 1998). Bid (−/−) mice are significantly protected from α-Fas-induced liver injury/lethality (Yin et al., 1999) and are therefore an essential component in the amplification of the apoptotic cascade. In these studies, Bid cleavage was inhibited by IDN-1965, theoretically due to caspase 8 inhibition, but it may also be due to the drug’s ability to inhibit caspase 3 (Woo et al., 1999).

Recent data also suggest that IDN-1965 is not only effective in protecting hepatocytes, but also sinusoidal endothelial cells. Natori et al. showed that IDN-1965 inhibited sinusoidal endothelial cell apoptosis in a model of cold ischemia/warm reperfusion (Natori et al., 1999). Sinusoidal cell apoptosis has been shown to be an early and critical event after liver transplantation (Gao et al., 1998). Indeed, it was also demonstrated that IDN-1965 increased the survival time of rats undergoing liver transplantation (Gao et al., 1998). Indeed, it was also demonstrated that IDN-1965 increased the survival time of rats receiving donor livers (Natori et al., 1999), suggesting that inhibition of apoptosis may prove beneficial in the transplantation process. In conclusion, this study supports the hypothesis that caspase inhibition may protect cells from abnormally large amounts of apoptosis seen in a number of disease states. Continual treatment with a caspase inhibitor may preserve the loss of critical numbers of cells responsible for the development of caspase-mediated cell death.

References


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