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Characterization of IDN-6556: a liver-targeted caspase inhibitor

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Abbreviations:

α-Fas: Anti-Fas agonistic antibody, ALT: Alanine aminotransferase, D-Gln/LPS: D-galactosamine/Lipopolysaccharide, DEVD-amc: Aspartyl-glutamyl-valinyl-aspartyl-aminomethylcoumarin, NADPH: Nicotinamide adenine dinucleotide phosphate, PAPS: 3'-Phosphoadenosine-5'-phosphosulfate, TNF-α: Tumor necrosis factor, UDPGA: Uridine diphosphate-glucuronic acid, ZVAD-fmk: Carbobenzyloxy-valinyl-alanyl-aspartyl-fluoromethylketone

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Abstract

The potency, efficacy and pharmacokinetic properties of IDN-6556, a first-in-class caspase inhibitor in clinical trials for the treatment of liver diseases, were characterized *in vivo* in rodent models. In the mouse α-Fas model of liver injury, IP administration of IDN-6556 resulted in marked reduction of ALT, apoptosis and caspase activities at a dose of 3 mg/kg. At this dose, IDN-6556 was also effective when given up to 2 hr prior to α-Fas and as late as 4 hr post α-Fas administration. In both the α-Fas and D-Gln/LPS model, ED_{50}s in the sub-mg/kg range were established after a number of routes of administration (IP, IV, IM, PO), ranging from 0.04 - 0.38 mg/kg. Efficacy was also demonstrated in the rat D-Gln/LPS model with 67 and 72 % reductions in ALT activities after IP and PO treatment with IDN-6556 (10 mg/kg), respectively. Pharmacokinetic analysis in the rat demonstrated rapid clearance after IV, IP and SC administration with terminal $t_{1/2}$ ranging from 46 to 51 minutes. Low absolute bioavailability after PO administration was seen (2.7-4%), but portal drug concentrations after oral administration were 3-fold higher than systemic concentrations with a 3.7-fold increase in the terminal $t_{1/2}$, indicating a significant first-pass effect. Liver concentrations remained constant after oral administration for at least a 4 hr period, reaching a C_{max} of 2,558 ng/g liver at 120 minutes. Lastly, 51 ± 20 and 4.9 ± 3.4% of IDN-6556 was excreted intact in bile after IV and PO administration, respectively. This evaluation indicates that IDN-6556 has marked efficacy in models of liver disease after oral administration and thus, is an excellent candidate for the treatment of liver diseases characterized by excessive apoptosis.

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Apoptosis, or programmed cell death, is characterized by distinctive morphological changes such as cell shrinkage and nuclear condensation. Biochemically, a hallmark of apoptosis is the activation of caspases, which are cysteine aspartyl proteases responsible for cleavage of a number of critical cellular targets (reviewed in Earnshaw et al. 1999). Activation of caspase 8 by apoptotic signaling pathways including Fas and TNF, in turn, activates other apical caspases (e.g. caspase 9) or "executioner" caspases (e.g. caspases 3, 6, and 7) that ultimately target a number of cellular components (Cohen, 1997). This caspase cascade results in the transformation of a functioning cell into an apoptotic body. While low levels of apoptosis normally occur to maintain homeostasis, abnormal amounts of apoptosis occur in disease states, especially in the liver (Galle, 1997).

Preclinically, a number of reports demonstrate that inhibition of caspases protected the liver from apoptosis-associated liver injury. Prototypical caspase inhibitors such as ZVAD-fmk were efficacious in a number of animal models including α-Fas and TNF-mediated liver injury (Rodriguez et al., 1996: Suzuki et al. 1998: Kunstle et al., 1997). More recently, other caspase inhibitors also were efficacious in rodent models of liver disease (Natori et al., 1999: Hoglen et al., 2001). Efficacy with these broad-spectrum caspase inhibitors preclinically suggests that they may have potential for the treatment of liver diseases in man. Abnormally high amounts of apoptosis have been reported in a number of liver diseases including alcoholic hepatitis (Kawahara et al., 1994), transplantation (Tannapfel et al., 1999) Wilson's disease (Strand, et al. 1998) and viral hepatitis (Lau et al. 1998).

This paper describes the preclinical efficacy and pharmacokinetics of a novel irreversible, broad-spectrum caspase inhibitor, IDN-6556. This drug is currently in Phase II clinical trials for liver diseases where it reduced plasma ALT activities in liver-impaired patients (Valentino et al., 2003). Due to its potency, efficacy and preferential distribution to the liver, the use of IDN-6556 for the treatment of liver diseases is warranted.

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Methods and Materials

Materials

Anti-Fas antibody (α-Fas) was purchased from PharMingen (Jo-2: PharMingen, San Diego, CA). Pentobarbital (Nembutol) was purchased from Western Medical Supply (Arcadia, CA). Lipopolysaccharide was purchased from List Biologicals (*E coli,* 0111.B4; Campbell, CA). D-Galactosamine was purchased from Calbiochem (San Diego, CA). Microsomes and S9 fractions were obtained from In Vitro Technologies (Baltimore, MD). All other materials were purchased from Sigma Chemical Co., (St. Louis, MO) unless indicated specifically in the text.

Animals and Treatment

Male balb/c mice (Harlan Sprague-Dawley, Indianapolis, IN) weighing approximately 20-25 grams were used for the α-Fas and D-Gln/LPS experiments. Male Sprague-Dawley rats weighing approximately 250 g (Harlan Sprague-Dawley, Indianapolis, IN) were used for the D-Gln/LPS and pharmacokinetic studies. For the biliary excretion experiments, bile duct-cannulated rats were purchased (Harlan Sprague-Dawley, Indianapolis, IN). All animals were acclimated for at least 3 days prior to experimentation.

α-Fas Model. α-Fas prepared in saline was injected via tail vein, iv at a dose of 200 μg/kg. IDN-6556 was prepared in either saline (when administered as a sodium salt), 100 mM phosphate buffer (pH 7.4) or DMSO:water (1:1) and dosed immediately after administration of α-Fas. Similar volumes of appropriate vehicle were administered to control animals. Preliminary experiments determined that the vehicles do not affect α-Fas- or D-Gln/LPS-mediated liver injury (data not shown). When comparing efficacy by multiple routes of administration, IDN-6556 was administered IP, PO, IM or IV (0.03 – 3 mg/kg) immediately after administration of α-Fas antibody. Six hr after administration of α-Fas, mice were anesthetized with pentobarbital (50 mg/kg, ip) and plasma was prepared from blood collected by cardiac puncture. In some experiments, livers were also harvested and immediately frozen for determination of caspase 3-like activity or formalin-fixed, embedded in paraffin and stained with hematoxylin and eosin for histological analysis. When IDN-6556 or saline was administered pre- or post-Fas, treatment

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times are indicated relative to α-Fas. In the post-dose experiment, lethality was monitored in a separate group of mice over a seven-day period.

D-Gln/LPS model. *Mice.* D-Gln (700 mg/kg) with 10 µg/kg LPS in saline were administered to mice. Four hr after administration, mice were treated with either vehicle or drug as in the Fas experiments. Ten hr after administration of D-Gln/LPS, mice were sacrificed: plasma was prepared from blood collected by cardiac puncture and in some experiments, caspase activity was determined in the harvested livers as described below. *Rats.* The rat studies were performed in the same manner as the mouse model except that 500 µg/kg of LPS and 500 mg/kg of D-Gln were used.

Pharmacokinetic experiments. IDN-6556 was prepared in a 100 mM sodium phosphate buffer at 10 mg/ml and dosed at a final volume of 1 ml/kg (10 mg/kg). For most pharmacokinetic studies, male Sprague-Dawley rats were cannulated in the carotid artery under isoflurane anesthesia and allowed to recover for at least one day before drug administration. Blood (100 µl/sample) was taken from the carotid cannula 2-240 minutes after administration of IDN-6556 (IV, SC, PO or IP). Serum was prepared and frozen immediately until analysis. In studies measuring drug concentrations in portal and systemic blood, individual rats were bled (3 animal/timepoint) simultaneously from the portal vein and inferior vena cava. In the biliary excretion study, bile was collected from the common bile duct after IV and PO administration of IDN-6556 (10 mg/kg) over a 24 hr period on ice and frozen until analysis.

Assays

ALT activities and histology. ALT activities in plasma or serum were determined using a standard diagnostic kit (Sigma). Portions of livers were fixed in 10% neutral buffered formalin, embedded in paraffin and stained with hematoxlin and eosin for morphological analysis.

Caspase Activity. Caspase activities in liver homogenates were determined as previously described in detail by monitoring the cleavage of DEVD-amc as a substrate (Hoglen et al., 2001).

Determination of IDN-6556 in serum and liver. Serum or bile samples were subjected to a liquid: liquid extraction with Methyl t-butyl ether. Methyl t-butyl ether containing IDN-6556 was evaporated to dryness and reconstituted with acetonitrile:1% formic acid in water (1:1). Samples were analyzed by LC/MS/MS with negative electrospray ionization. Concentrations were determined based on a standard curve ranging from 0 – 5000 ng/ml. The limit of quantitation

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(LOQ) was 5 ng/ml. For determination of hepatic concentrations of IDN-6556, livers were homogenized in acetonitrile, centrifuged and then the supernatants were analyzed. Data are expressed as ng drug/gram of liver extracted.

In vitro metabolism. Microsomes (1 mg total protein) with a NADPH generating system (NADP, glucose-6-phosphate, glucose-6-phophate dehydrogenase, NADPH and $MgCl_2$) and S9 fractions (1 mg total protein: containing 100 µg/ml PAPS and 1.9 mg/ml UDPGA) were incubated for 45 minutes at 37 ^{o}C in the presence of 100 µM IDN-6556 or testosterone. Incubations were stopped by addition of cold acetonitrile. Samples were centrifuged and the resulting supernatants were analyzed for IDN-6556 or testosterone by HPLC. All analyses were performed in triplicate. Data are expressed as the % of drug metabolized (1 - ratio of drug in active/ denatured protein) *100%.

Statistical Analyses. For efficacy studies, data are expressed as mean $\pm$ standard error of the mean (SEM). ED_{50}s with 95% confidence intervals were calculated by plotting ALT activities expressed as $(ALT_{\alpha\text{-Fas/drug})} - ALT_{vehicle})/(ALT_{\alpha\text{-Fas/vehicle}}-ALT_{vehicle})$ x 100%, followed by non-linear regression analysis using GraphPad Prism Software, Inc. (San Diego, CA). When comparing differences between groups, a One-Way Analysis of Variance was performed followed by a Student Newman-Kuels post-hoc test. Differences were considered significant when $p < 0.05$. For pharmacokinetic studies, data are expressed as mean $\pm$ standard deviation of the mean (SD). Serum or liver concentrations of IDN-6556 obtained from LC/MS/MS analysis were subjected to a non-compartmental pharmacokinetic analysis using WinNonlin software (V.3.01: Pharsight, Inc., Mountain View, CA.).

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RESULTS

IDN-6556 was initially tested in the α-Fas model of liver injury, a model characterized by lethality, marked hepatocellular apoptosis, and peak ALT activities within 6 hr (Ogasawara et al., 1993). When drug was administered IP immediately after administration of α-Fas, ALT activities, measured 6 hr later, decreased in a dose-dependent manner with an ED_{50} of 0.08 (0.06 – 0.12) mg/kg (Figure 1A). At 6 hr, marked hepatocellular apoptosis was seen histologically after α-Fas administration and the apoptosis was dose-dependently reduced with IDN-6556 (data not shown). Caspase 3-like activities, measured as DEVD-amc cleavage, dose-dependently decreased with a 92.5 % reduction following the highest dose of IDN-6556 (3 mg/kg, Figure 1B). When examined at 24 hr after α-Fas administration, caspase activity was still elevated in the α-Fas treated mice (0.15 ± 0.04 Δrfu/μg/hr). However, at this time point, treatment with IDN-6556 (3 mg/kg) did not reduce caspase activity (0.15 ± 0.03 Δrfu/μg/hr), indicating that the inhibitory effect was transient. To determine the effect of routes of administration, the potency of IDN-6556 after IV, PO and IM administration were compared: ED_{50}s of 0.38 (0.11 – 1.27), 0.31 (0.24 – 0.42) and 0.04 (0.02 – 0.07) mg/kg were determined in the α-Fas model, respectively (Table 1). The effect of pre- and post-administration of IDN-6556 was then evaluated in the α-Fas model. Pre-administration of IDN-6556 (3 mg/kg, IP) immediately or 2 hr prior to α-Fas resulted in significant reductions in ALT activities, whereas administration of IDN-6556 4 or 6 hr prior did not (Figure 2A). In contrast, drug was effective when dosed IP as late as 4 hr post α-Fas administration, indicating post-insult efficacy (Figure 2B). Further, in this study, 40 % of mice died in the control group (4/10) by 24 hr, whereas no mice receiving α-Fas and IDN-6556 (3 mg/kg, 0-4 hr, IP) died within 7 days, regardless of the time of IDN-6556 administration.

IDN-6556 was then tested in the D-Gln/LPS model, a murine model also characterized by marked rises in ALT activities and apoptosis within 6-8 hr (Mignon et al. 1999). When IDN-6556 was given four hr after D-Gln/LPS administration to mice and ALT activities were determined in plasma 10 hr after D-Gln/LPS administration, ED_{50}s after IP and IV administration of IDN-6556 were 0.17 (0.09 – 0.32) mg/kg and 0.09 (0.03 – 0.31) mg/kg, respectively (Table 1). Further, after IP administration, caspase activity was reduced by 74% at doses of 1 mg/kg or higher (data not

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shown). When drug was administered orally in two independent experiments, an ED_{50} could not be determined since the lowest dose of IDN-6556 (0.01 mg/kg) reduced ALT activities by over 50%.

After oral administration of IDN-6556 in the rat D-Gln/LPS model, ALT activities were dose-dependently reduced with decreases ranging from a 47 – 76% (ED_{50} of 1.2 mg/kg; Figure 3). A 67% reduction in ALT activities was seen after an IP dose of IDN-6556 (10 mg/kg): and caspase activities were reduced by 57% with the 10 mg/kg of IDN-6556. Lastly, three of 14 vehicle-treated rats died from D-Gln/LPS administration, but there was no lethality in any IDN-6556-treated rats that also received D-Gln/LPS.

Pharmacokinetics of IDN-6556 were determined in rats. After a single bolus administration (IV, IP or SC), IDN-6556 had terminal half-lives of 51 ± 11, 47 ± 5, 46 ± 8 minutes, respectively (Figure 4, Table 2). Bioavailability was 49 and 70% for IP and SC administration, respectively. In two separate experiments, oral bioavailability of IDN-6556 (10 mg/kg, in fasted rats) was low, ranging from 2.7 to 4%. Representative pharmacokinetic profiles are shown in Figure 4.

Because apparent oral bioavailability of IDN-6556 in the rat was low, but efficacy was similar after IP and PO administration, portal and systemic concentrations of IDN-6556 after both IV and PO administration were compared. In rats treated IV with IDN-6556, pharmacokinetic profiles and parameters in serum from the portal and systemic compartments were similar (Table 3). After oral administration, AUC_{inf} and MRT_{inf} of IDN-6556 in the portal vein were 5.9 and 5.3-fold higher, respectively, than in the systemic compartment, suggesting a marked first-pass effect (Table 3). In a separate study, portal vein and extractable liver concentrations of IDN-6556 remained elevated over the 4 hr period reaching 2,560 ng/g liver at 120 minutes and a terminal $t_{1/2}$ in liver could not be determined within 4 hr. IDN-6556 concentrations were greatest in the liver>>portal>systemic blood. Pharmacokinetic profiles for portal and liver concentrations suggest the occurrence of enterohepatic recirculation due to a secondary rise in IDN-6556 concentrations in the liver and portal system (Figure 5).

Cumulative biliary excretion after IV and PO administration of IDN-6556 was assessed. After IV administration, 51 ± 20 % (n=4) of an administered dose of IDN-6556 (10 mg/kg) was found intact in bile after 24 hr with the majority excreted within one hr. However, only 4.9 ± 3.5 %

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(n=3) was found in bile after PO administration of IDN-6556 (38% of the available dose: data not shown).

Lastly, stability of IDN-6556 incubated with rat microsomes and S9 fractions was assessed. In microsomes, only 4% of IDN-6556 was metabolized versus 85% for testosterone (Table 4). Similarly, in S9 fractions with or without addition of UDPGA and PAPS, only 5% of IDN-6556 was metabolized, indicating lack of phase II metabolism of IDN-6556 in rats.

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DISCUSSION

Excessive apoptosis within the liver is thought to lead to dysfunction and damage. Marked apoptosis was demonstrated in a variety of liver diseases including alcoholic hepatitis, biliary atresia and primary non-function in transplanted livers (Galle and Krammer, 1998). In these liver diseases, programmed cell death appears to play a causal role either in the initiation and/or progression of the disease. Apoptotic pathways including Fas or TNF-α are up-regulated, and ultimately the activation of caspases occurs. Pan-inhibition of caspases therefore may provide an excellent strategy to halt the apoptotic signal, which would enhance cell survival. This approach was proven preclinically as caspase inhibitors were effective in eliminating liver injury/lethality in various models of liver injury.

This report characterizes in rodents, the efficacy and pharmacokinetics of a novel pan-caspase inhibitor in clinical development, IDN-6556. IDN-6556 selectively and irreversibly inhibited activated caspases with IC_{50}s in the low to sub-nanomolar range against caspases 1, 3, 6, 7, 8 and 9 as well as sub-micromolar efficacy in a variety of cellular assays (Kodandapani et al., unpublished). Preclinically, IDN-6556 was effective in inhibiting apoptosis of sinusoidal endothelial cells in a rat model of cold-ischemia/warm reperfusion (Natori et al., 2003). This drug is a first-in-class anti-apoptotic caspase inhibitor with demonstrated preliminary efficacy in liver-impaired patients in humans (Valentino et al., 2003).

The first goal of this study was to determine efficacy and potency of IDN-6556 in models characterized by caspase-mediated apoptosis initiated by the activation of apoptotic signals such as Fas and TNF-α. In both the mouse α-Fas and D-Gln/LPS models, IDN-6556 demonstrated marked potency by a number of routes of administration when given simultaneously as well as post-insult. Further, the compound appeared to act mechanistically by inhibiting caspase activities at the same doses that decreased plasma ALT activities. These decreases in ALT and caspase activities were dose-dependent, with maximal reductions of 90% in the Fas model and 74% in the D-Gln/LPS model. Plasma ALT activities were reduced to control levels with doses as low as 1 mg/kg. Histological analysis confirmed the protection: livers from α-Fas-treated mice that were administered IDN-6556 (3 mg/kg) had no or mild signs of liver injury when examined 6 hours later. These data confirm reports using broad-spectrum caspase inhibitors in models of

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liver injury, including the prototypical caspase inhibitor ZVAD-fmk (Rodriguez et al, 1996) as well as the recently characterized IDN-1965 (Hoglen et al., 2001). Importantly, IDN-6556 inhibited Fas-mediated liver injury and lethality when administered up to 4 hr after α-Fas, demonstrating post-insult efficacy. While liver injury was not totally inhibited when compound was administered this late, lethality was completely blocked. Lack of complete efficacy is not surprising after post-insult since caspase 3-like activity was already maximal by 4 hr and significant injury had already occurred (Hoglen et al., 2001). The post-insult efficacy was confirmed in the D-Gln/LPS model as dosing of IDN-6556 is effective 4 hr after administration of D-Gln/LPS, well after TNF-α had reached peak levels (Bahrami et al., 1994). Therefore, the data suggest that this drug can still be effective when administered post-insult in clinical situations. In contrast, pre-administration resulted in protection 2 hr, but not 4 hr, prior to α-Fas administration, suggesting that the compound's biological activity is temporary and reversible. Additional support for this hypothesis is that the caspase activity was blocked 6 hr, but not 24 hr after α-Fas administration (data not shown).

One interesting difference observed in the D-Gln/LPS model was that the potency and protective effect of IDN-6556 was better in mice than rats. For example, after oral administration, the ED_{50} of less than 0.01 mg/kg was determined in the mouse but only 1.2 mg/kg in the rat. Further, while near complete reduction in ALT activities were evident in the mouse models with doses of 1 mg/kg or less, maximal reduction in the rat with a 10 mg/kg dose of IDN-6556 was 76%. Reasons for the difference in efficacy is not known, however, other studies demonstrate that caspase independent necrosis occurs in the rat model of D-Gln/LPS (Stachlewitz et al, 1999; Gujral et al, 2003). In fact, in the study by Gujral et al., a similar caspase inhibitor was effective in reducing caspase activities to control levels, but only modestly lowered ALT activities, depending on time of measurement. In contrast, as in our study, previous studies demonstrate that ZVAD affords near complete protection in the mouse D-Gln/LPS model (Jaeschke et al., 1998). Collectively, the decreased potency in the rat may be due to a greater caspase independent component of hepatocellular cell death in the rat.

In general, potency of IDN-6556 after oral dosing in the α-Fas and D-Gln models was similar to other routes of administration. In fact, in the murine D-Gln/LPS model, the ED_{50} of IDN-6556 was less than 0.01 mg/kg after oral administration and the drug was most potent by this

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route of administration. Comparable activity was seen in the rat where efficacy after PO administration was similar to IP administered IDN-6556. Further, lethality was completely inhibited at the lowest dose of IDN-6556 in the rat D-Gln/LPS model (1 mg/kg). Collectively, the efficacy studies suggest that exposure of liver to IDN-6556 was similar by all routes of administration in both rats and mice. To further investigate exposure, the pharmacokinetic profiles of IDN-6556 were assessed following systemic and oral routes of administration to determine bioavailability by various routes of exposure. Single doses of IDN-6556 were cleared relatively quickly as seen by a high clearance rates as well as terminal $t_{1/2}$ of less than 60 minutes following IV, IP and SC administration. These data support the findings in the prophylactic studies in mice where IDN-6556 in the Fas model was not efficacious when administered greater than 2 hrs prior to α-Fas administration. Biological activity appears to last approximately 6-8 hours after administration, which coincides with nearly complete elimination. Surprisingly, however, oral administration resulted in bioavailability of only 4.0%. For example, in the rat, the AUC_{inf} after IP administration was 15-fold greater than after oral administration, suggesting that oral potency would be less. To determine a possible explanation for this finding, the pharmacokinetics of the drug in portal and systemic blood following IV and PO administration were characterized to determine whether there were higher portal concentrations after oral administration that could result in greater exposure to the liver than would be predicted from systemic blood levels. Indeed, the AUC_{last} and AUC_{inf} of IDN-6556 after PO administration in portal plasma compared to systemic concentrations was approximately 4 and 6-fold greater, respectively. Further, the $t_{1/2}$ and MRT_{last} were also 4.7 and 3-fold higher in the portal system, suggesting that the compound was undergoing first pass effect and increasing exposure to the liver but not to other organs. Even after 4 hours, portal and liver concentrations after PO administration were nearly at maximal levels. This was contrary to the pharmacokinetics seen in portal and systemic plasma following IV administration, where most of the drug was eliminated by 4 hr.

Rapid elimination of the drug appeared to be, at least in part, due to biliary excretion. In the rat, bile contained over 50% of the intact drug after IV administration, and most of the excreted drug was found within the first hour. Mechanisms by which the drug was excreted are currently unknown, but there are multiple mechanisms that eliminate negatively charged small molecules including organic anion transporter proteins into the bile (Meier et al., 1997). In

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contrast, rapid elimination of the drug did not appear to involve cytochromes P-450 mediated metabolism, as only a small percentage of IDN-6556 was metabolized by microsomes in the rat. Additionally, significant phase II metabolism appears unlikely as there was little metabolism in S-9 fractions.

Thus, oral administration resulted in adequate drug concentrations to the liver as evidenced by oral potency similar to that of other routes of administration. In contrast, systemic exposure of IDN-6556 after oral administration was low, as determined by a small AUC and C_{max}. This may have advantages with respect to toxicology of the drug, since exposure to organs other than the liver and GI tract would be lower following oral administration. It also has the advantage of site-directed targeting since the current indications for this drug are diseases of the liver with excessive apoptosis such as alcoholic hepatitis and recipients of liver transplantation.

In sum, this report shows that the broad-spectrum caspase inhibitor, IDN-6556 was potent in models of liver injury in both the mouse and rat, due to reduction in apoptosis via inhibition of caspases. IDN-6556 was as effective orally as by other routes of administration, despite low absolute oral bioavailability and rapid systemic elimination. However, the drug appeared to have high entero-hepatic recirculation and first pass effect as evidenced by a secondary peaks in liver and plasma as well as relatively high portal levels compared to systemic concentrations after oral administration. In conclusion, IDN-6556 is an excellent candidate for the treatment of liver diseases characterized by excessive apoptosis.

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Figure legends.

Figure 1. Dose-dependent inhibition of α-Fas-induced liver injury and caspase activity by IDN-6556. Mice were administered α-Fas (200 µg/kg, IV) immediately followed by IDN-6556 (0.03 – 3.0 mg/kg, IP). Mice were killed 6 h later and then blood and livers were harvested. Plasma was prepared for measurement of ALT activities. **A**. ALT activities. ALT activities are expressed as $(ALT_{\alpha\text{-Fas/drug}}) - ALT_{vehicle})/(ALT_{\alpha\text{-Fas/vehicle}}-ALT_{vehicle}) \times 100\%$. ED_{50} is expressed with 95% confidence limits in parentheses. N = 6-10/group. **B**. Caspase activities. Data are expressed as mean ± sem. N=6-10/group. Control value is basal caspase activity in vehicle-treated controls.

Figure 2. Pre- and post dose efficacy by IDN-6556 in the α-Fas model. A. Saline or IDN-6556 (3.0 mg/kg, IP in saline) was administered to mice 6, 4, 2 or 0 hr prior to α-Fas (200 µg/kg, IV). **B.** α-Fas (200 µg/kg, IV) was administered to mice followed by IDN-6556 (3.0 mg/kg, IP) 0, 2, 3 or 4 hr later. Mice were euthanized 6 hr after α-Fas and blood was harvested. Plasma was prepared for measurement of ALT activities. ALT activities are expressed as percent of α-Fas controls. N=5-13/group. *Indicates a significant reduction from the α-Fas treated control ($p<0.05$).

Figure 3. Reduction of D-Gln/LPS-induced liver injury in rats by IDN-6556. Rats received 500 mg/kg D-Gln with 500 µg/kg LPS, IP, dissolved in saline. Four hr after administration of D-Gln/LPS, rats were treated with either vehicle or drug (1, 3 and 10 mg/kg, PO or 10 mg/kg, IP). Ten hr after D-Gln/LPS administration, rats were sacrificed and plasma was prepared from harvested blood. ALT activities were determined in plasma and expressed as mean ± sem. N=6-8/group. *Indicates significant difference from D-Gln/LPS treated control ($p<0.05$).

Figure 4. Pharmacokinetic profiles of IDN-6556 after four routes of administration. Carotid artery-cannulated rats received IDN-6556 (10 mg/kg) prepared in 100 mM phosphate buffer by IV, IP, SC or PO administration. Blood was taken from the carotid cannula 2 –240 minutes after drug administration. Plasma prepared from blood was extracted and IDN-6556 was quantified by LC/MS/MS as described in Methods. Data are expressed as mean ± SD. N=3-4/group.

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Figure 5. Systemic, portal and liver concentration-time curves of IDN-6556 after oral administration in rats. IDN-6556 prepared in 100 mM phosphate buffer was administered PO to rats (10 mg/kg). Blood was taken via the portal vein and inferior vena cava and livers were harvested 2-240 minutes after dose administration. Blood and livers were analyzed by LC/MS/MS for IDN-6556 as described in Methods. Data are represented as mean ± SD. N=3/timepoint.

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Figure 1

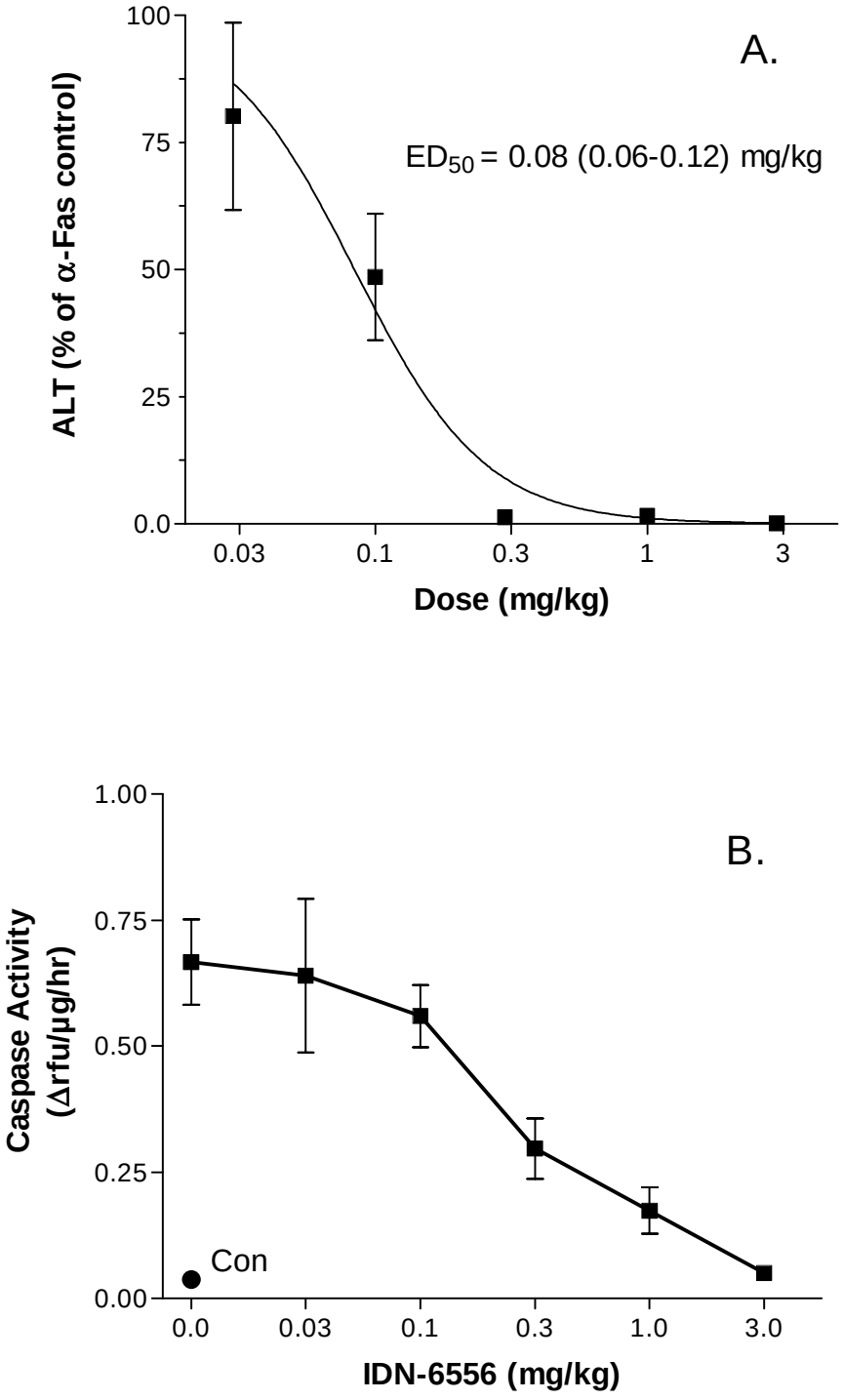

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Figure 2

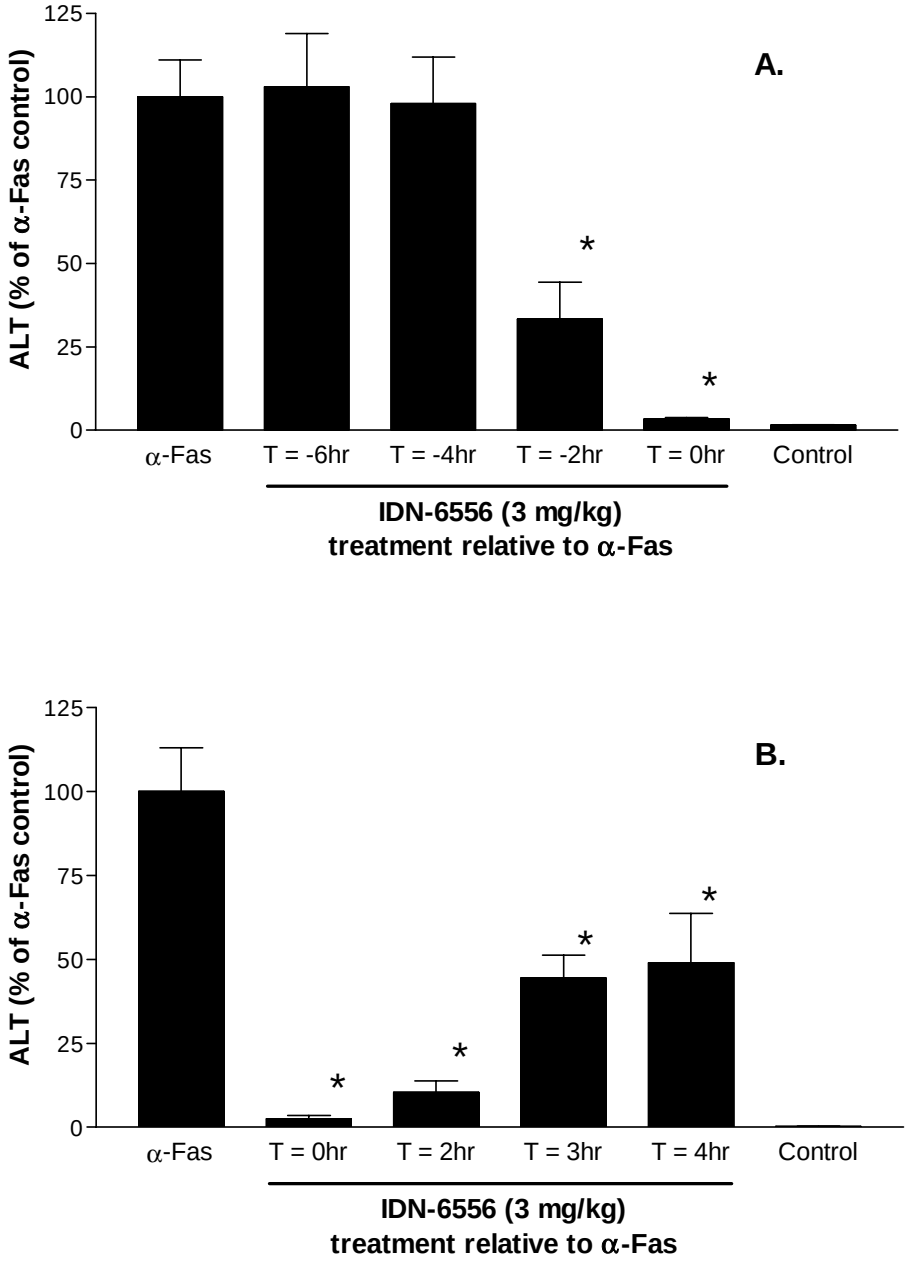

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Figure 3

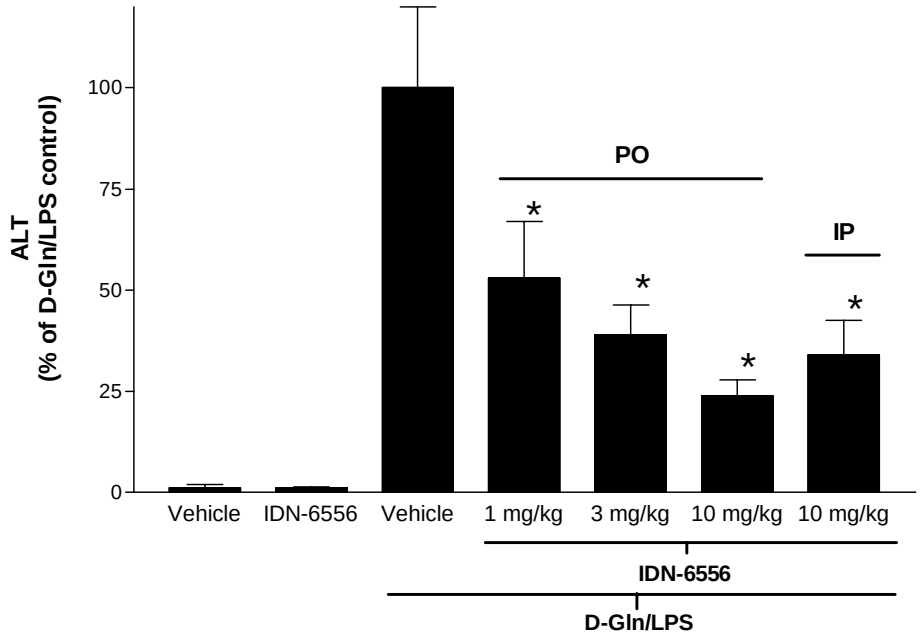

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Figure 4

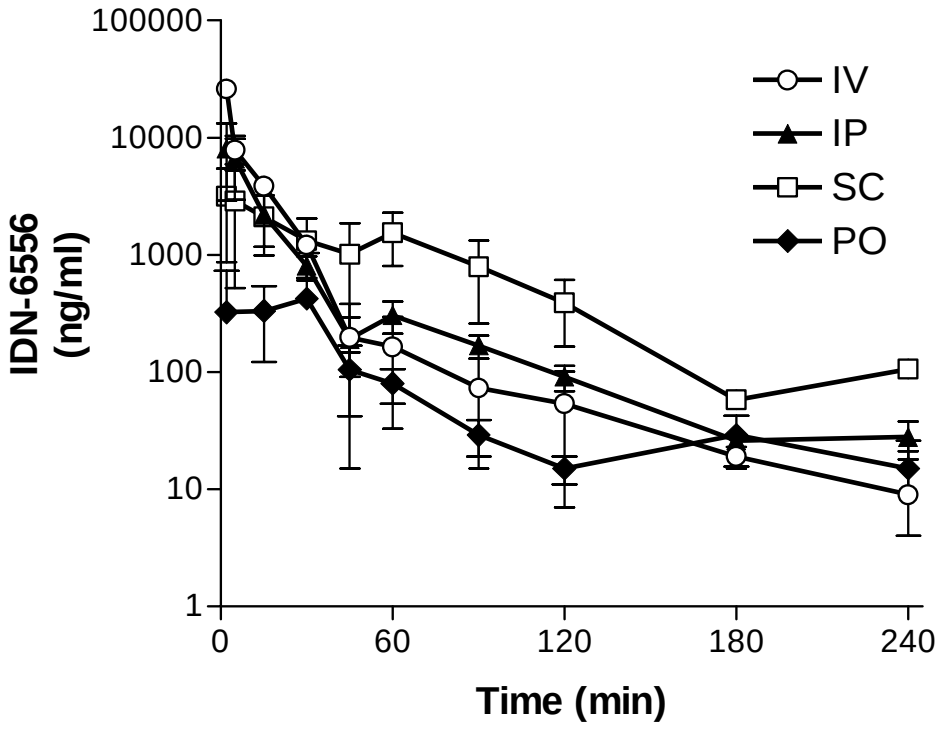

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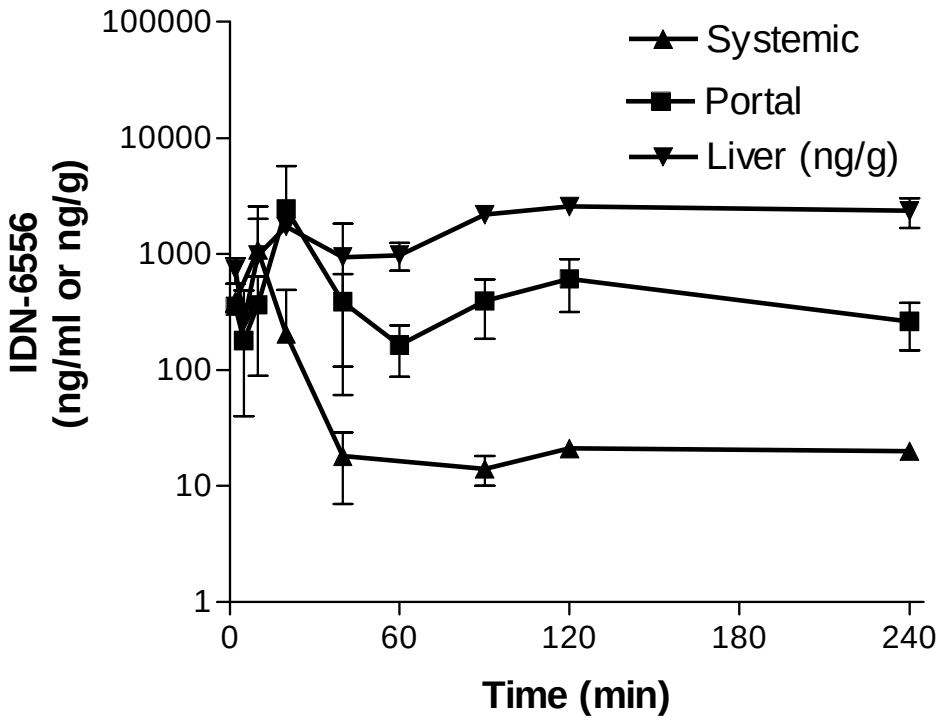
Figure 5



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Model	Route of Administration	ED_{50} (mg/kg)	Confidence Intervals (mg/kg)
α-Fas	IP	0.08	0.06 - 0.12
α-Fas	IV	0.38	0.11 – 1.27
α-Fas	PO	0.31	0.24 – 0.42
α-Fas	IM	0.04	0.02 - 0.07
D-Gln/LPS	IP	0.17	0.09 – 0.32
D-Gln/LPS	IV	0.09	0.03 – 0.31
D-Gln/LPS	PO	<0.01	---

Table 1. Potency of IDN-6556 in two murine models of liver injury. *α-Fas:* Mice were administered α-Fas (200 µg/kg, IV) immediately followed by IDN-6556 (0.03 – 3.0 mg/kg). Mice were euthanized 6 h later and blood was taken via cardiac puncture. ALT activities measured in plasma and are calculated as $(ALT_{\alpha\text{-Fas/drug}} - ALT_{vehicle})/ALT_{\alpha\text{-Fas/vehicle}}\text{-}ALT_{vehicle}) \times 100\%$. ED_{50}s are expressed with 95% confidence limits in parentheses. N = 6-10/group.
D-Gln/LPS: Balb/c mice received D-Gln and LPS (700 mg/kg and 10 µg/kg, respectively), by IP injection dissolved in saline (5 ml/kg dosing volume). Four hours post administration of D-Gln/LPS, vehicle or IDN-6556 (0.01 – 3.0 mg/kg) was given by the specified route. Ten hr after administration of D-Gln/LPS, mice were euthanized with pentobarbital (50 mg/kg, IP), plasma was harvested by cardiac puncture and ALT activities were determined. Data are expressed as % ALT of D-Gln/LPS-treated controls.

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Parameters	Units	IV	IP	SC
T_{max}	(min)	0	2 ± 0	2 ± 0
C_{max}	(ng/ml)	59,000 ± 9,000	8,100 ± 5,20	3,20 ± 2,300
$t_{1/2}$	(min)	51 ± 11	47 ± 5	46 ± 8
AUC_{last}	(ng*hr/ml)	4,200 ± 320	2,100 ± 520	2,900 ± 1,70
AUC_{inf}	(ng*hr/ml)	4,300 ± 320	2,100 ± 500	3,000 ± 1,600
V_{ss}	(l/kg)	0.45 ± 0.09	N/A	N/A
CL	(ml/kg/min)	39 ± 3	N/A	N/A
MRT_{inf}	(min)	12 ± 3	30 ± 9	69 ± 11
% F	(%)	100	49	70

Table 2. Pharmacokinetic parameters for IDN-6556 after various routes of administration. IDN-6556 (10 mg/kg) was administered IV, IP, SC or PO to rats in 100 mM phosphate buffer (pH 7.4). Serum samples were prepared from blood taken via carotid artery 2, 5, 15, 30, 45, 60, 90, 120, 180, and 240 minutes after administration and analyzed by LC/MS/MS as described in the methods. Data are expressed as mean ± SD. N=3-4 rats/timepoint. Bioavailability was calculated from the AUC_{inf}.

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	T_{max} (min)	C_{max} (ng/ml or ng/g)	$t_{1/2}$ (min)	AUC_{last} (ng*hr/ml or ng*hr/g)	AUC_{inf} (ng*hr/ml or ng*hr/g)	V_{ss} (l/kg)	CL_{last} (ml/kg/min) apparent	MRT_{last} (min)	MRT_{inf} (min)
PO									
Liver	120	2,600	ND*	8,000	ND*	2,900	--	137	ND*
Portal	20	1030	120	540	790	--	--	88	191
Systemic	10	600	32	130	130	--	--	31	36
IV									
Portal	0	69,000	50	4,300	4,300	850	39	20	22
Systemic	0	64,000	49	3,500	3,500	680	47	13	14

%F Systemic: AUC_{po}/AUC_{iv} x 100% = 4%

%F Portal: AUC_{po}/AUC_{iv} x 100% = 12%

*Could not be determined.

Table 3. Systemic, portal vein, liver concentrations-time curves of IDN-6556 after PO and IV administration in rats. IDN-6556 prepared in 100 mM phosphate buffer was administered PO or IV to rats (10 mg/kg). Serum samples prepared from blood taken via the portal vein and inferior vena cava ranging from 2 to 240 minutes. IDN-6556 concentrations were quantified in sera and harvested livers by LC/MS/MS as described in the methods. Data are represented as mean ± SD. N=3 for liver and n=6 portal and systemic concentrations.

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	% Reduction of drug (ratio of active/denatured protein)		
Drug	**Microsomes**	**S9**	**S9 w/ UDPGA and PAPS**
IDN-6556	4	0	5
Testosterone	85	74	56

Table 4. Microsomal and S9 stability of IDN-6556. IDN-6556 (50 µM) or testosterone (50 µM) were incubated with microsomes (active or heat-denatured) or S9 (active or heat-denatured) supplemented with UDPGA (2 mg/ml) and PAPS (100 µg/ml) for 45 minutes at 37^0C. The reaction was stopped with addition of acetonitrile. Compounds were extracted with acetonitrile and analyzed by HPLC.

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