

Characterization of IDN-6556 (3-{2-(2-*tert*-Butyl-phenylaminoxyalyl)-amino]-propionylamino}-4-oxo-5-(2,3,5,6-tetrafluoro-phenoxy)-pentanoic Acid): a Liver-Targeted Caspase Inhibitor

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ABSTRACT

The potency, efficacy, and pharmacokinetic properties of IDN-6556 (3-{2-[(2-*tert*-butyl-phenylaminoxyalyl)-amino]-propionylamino}-4-oxo-5-(2,3,5,6-tetrafluoro-phenoxy)-pentanoic acid), 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, i.p. administration of IDN-6556 resulted in marked reduction of alanine aminotransferase (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 h before α -Fas and as late as 4 h after α -Fas administration. In both the α -Fas and D-galactosamine/lipopolysaccharide (D-Gln/LPS) model, ED₅₀ values in the sub-milligram per kilogram range were established after a number of routes of administration (i.p., i.v., i.m., or p.o.), ranging from 0.04 to 0.38 mg/kg. Efficacy was also demonstrated in the rat D-Gln/LPS model with 67 and 72% reductions in ALT activities

after i.p. and p.o. treatment with IDN-6556 (10 mg/kg), respectively. Pharmacokinetic analysis in the rat demonstrated rapid clearance after i.v., i.p., and s.c. administration with terminal $t_{1/2}$ ranging from 46 to 51 min. Low absolute bioavailability after p.o. 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-h period, reaching a C_{max} of 2558 ng/g liver at 120 min. Last, 51 ± 20 and 4.9 ± 3.4% of IDN-6556 was excreted intact in bile after i.v. and p.o. 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.

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 several critical cellular targets (for review, see 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. Although low levels of apoptosis normally occur to maintain homeostasis, abnormal amounts of apoptosis occur in disease states, especially in the liver (Galle, 1997).

Preclinically, several reports demonstrate that inhibition of caspases protected the liver from apoptosis-associated liver injury. Prototypical caspase inhibitors such as ZVAD-FMK were efficacious in many animal models, including α -Fas- and TNF-mediated liver injury (Rodriguez et al., 1996; Kunstle et al., 1997; Suzuki, 1998). 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

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ABBREVIATIONS: TNF, tumor necrosis factor; ALT, alanine aminotransferase; α -Fas, anti-Fas agonistic antibody; D-Gln/LPS, D-galactosamine/lipopolysaccharide; LPS, lipopolysaccharide; LC/MS/MS, liquid chromatography tandem mass spectrometry; rfu, relative fluorescence unit; MRT, mean residence time; UDPGA, uridine diphosphate-glucuronic acid; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; AUC, area under the curve; DEVD-AMC, aspartyl-glutamyl-valinyl-aspartyl-aminomethylcoumarin; ZVAD-FMK, carbobenzyloxy-valinyl-alanyl-aspartyl-fluoromethylketone; IDN-6556, 3-{2-[(2-*tert*-butyl-phenylaminoxyalyl)-amino]-propionylamino}-4-oxo-5-(2,3,5,6-tetrafluoro-phenoxy)-pentanoic acid.

suggests that they may have potential for the treatment of liver diseases in humans. Abnormally high amounts of apoptosis have been reported in several 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 article 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.

Methods and Materials

Materials

Anti-Fas antibody (α -Fas) was purchased from BD PharMingen (San Diego, CA) (Jo-2). Pentobarbital (Nembutol) was purchased from Western Medical Supply (Arcadia, CA). Lipopolysaccharide was purchased from List Biologicals (Campbell, CA) (*Escherichia coli*, 0111.B4). 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-Aldrich (St. Louis, MO) unless indicated specifically in the text.

Animals and Treatment

Male BALB/c mice (Harlan, Indianapolis, IN), weighing approximately 20 to 25 g, were used for the α -Fas and D-Gln/LPS experiments. Male Sprague-Dawley rats, weighing approximately 250 g (Harlan), 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 before experimentation.

α -Fas Model. α -Fas prepared in saline was injected via tail vein, i.v., at a dose of 200 μ g/kg. IDN-6556 was prepared in either saline (when administered as a sodium salt), 100 mM phosphate buffer (pH 7.4), or dimethyl sulfoxide/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 i.p., p.o., i.m., or i.v. (0.03–3 mg/kg) immediately after administration of α -Fas antibody. Six hours after administration of α -Fas, mice were anesthetized with pentobarbital (50 mg/kg i.p.), 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 times are indicated relative to α -Fas. In the postdose experiment, lethality was monitored in a separate group of mice over a 7-day period.

D-Gln/LPS Model. For mice, D-Gln (700 mg/kg) with 10 μ g/kg LPS in saline were administered to mice. Four hours after administration, mice were treated with either vehicle or drug as in the Fas experiments. Ten hours 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. For rats, studies were performed in the same manner as the mouse model, except that 500 μ g/kg LPS and 500 mg/kg 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 1 day before drug administration. Blood (100 μ l/sample) was taken from the carotid cannula 2 to 240 min after administration of IDN-6556 (i.v., s.c., p.o., or i.p.). Serum was prepared and frozen immediately until analysis. In studies measuring drug concentrations in portal and systemic blood, individual rats were bled (three animals per time point) simultaneously from the portal vein and inferior vena cava. In the biliary excretion study, bile was collected from the common bile duct after i.v. and p.o. administration of IDN-6556 (10 mg/kg) over a 24-h 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-Aldrich). Portions of livers were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin for morphological analysis.

Caspase Activity. Caspase activities in liver homogenates were determined as described previously 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 to 5000 ng/ml. The limit of quantitation 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 nanograms of drug per gram of liver extracted.

In Vitro Metabolism. Microsomes (1 mg of total protein) with a NADPH-generating system (NADP, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, NADPH, and MgCl₂) and S9 fractions (1 mg of total protein: containing 100 μ g/ml PAPS and 1.9 mg/ml UDPGA) were incubated for 45 min at 37°C in the presence of 100 μ M 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 high-performance liquid chromatography. All analyses were performed in triplicate. Data are expressed as the percentage of drug metabolized (1 – ratio of drug in active/denatured protein) · 100%.

Statistical Analyses. For efficacy studies, data are expressed as mean \pm S.E.M. ED₅₀ values with 95% confidence intervals were calculated by plotting ALT activities expressed as $(ALT_{\alpha\text{-Fas}/\text{drug}} - ALT_{\text{vehicle}})/(ALT_{\alpha\text{-Fas}/\text{vehicle}} - ALT_{\text{vehicle}}) \times 100\%$, followed by nonlinear regression analysis using GraphPad Prism (GraphPad 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 S.D. Serum or liver concentrations of IDN-6556 obtained from LC/MS/MS analysis were subjected to a noncompartmental pharmacokinetic analysis using WinNonlin software (version 3.01; Pharsight, Mountain View, CA).

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 h (Ogasawara et al., 1993). When drug was administered i.p. immediately after administration of α -Fas, ALT activities,

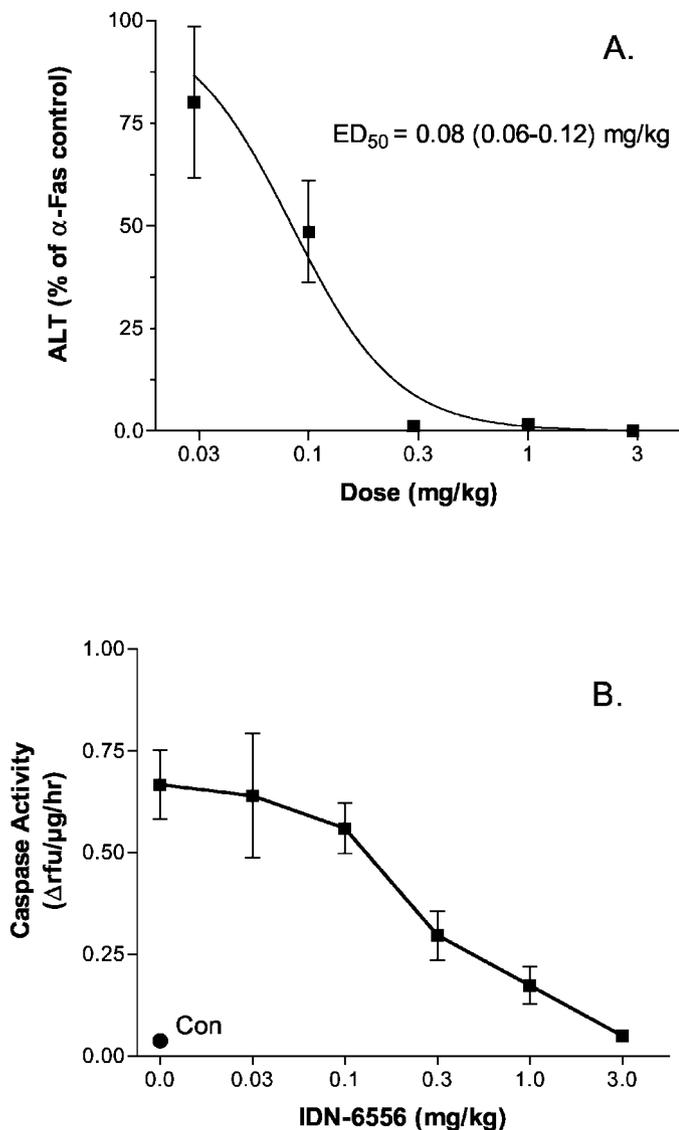


Fig. 1. Dose-dependent inhibition of α -Fas-induced liver injury and caspase activity by IDN-6556. Mice were administered α -Fas (200 μ g/kg i.v.) immediately followed by IDN-6556 (0.03–3.0 mg/kg i.p.). Mice were killed 6 h later and then blood and livers were harvested. Plasma was prepared for measurement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

measured 6 h later, decreased in a dose-dependent manner with an ED_{50} value of 0.08 (0.06–0.12) mg/kg (Fig. 1A). At 6 h, 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 after the highest dose of IDN-6556 (3 mg/kg; Fig. 1B). When examined at 24 h after α -Fas administration, caspase activity was still elevated in the α -Fas-treated mice (0.15 ± 0.04 Δ rfu/ μ g/h). However, at this time point, treatment with IDN-6556 (3 mg/kg) did not reduce caspase activity (0.15 ± 0.03 Δ rfu/ μ g/h), indicating that the inhibitory effect was transient. To determine the effect of routes of administration, the potency

TABLE 1

Potency of IDN-6556 in two murine models of liver injury

α -Fas: Mice were administered α -Fas (200 μ g/kg i.v.) 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 $(ALT_{\alpha\text{-Fas}/\text{drug}} - ALT_{\text{vehicle}})/ALT_{\alpha\text{-Fas}/\text{vehicle}} - ALT_{\text{vehicle}} \times 100\%$. ED_{50} values are expressed with 95% confidence limits in parentheses. $n = 6$ to 10/group.

D-Gln/LPS: BALB/c mice received D-Gln and LPS (700 mg/kg and 10 μ g/kg, respectively), by i.p. injection dissolved in saline (5 ml/kg dosing volume). Four hours postadministration of D-Gln/LPS, vehicle, or IDN-6556 (0.01–3.0 mg/kg) was given by the specified route. Ten hours after administration of D-Gln/LPS, mice were euthanized with pentobarbital (50 mg/kg i.p.), plasma was harvested by cardiac puncture, and ALT activities were determined. Data are expressed as percentage of ALT of D-Gln/LPS-treated controls.

Model	Route of Administration	ED_{50}	Confidence Interval
<i>mg/kg</i>			
α -Fas	i.p.	0.08	0.06–0.12
α -Fas	i.v.	0.38	0.11–1.27
α -Fas	p.o.	0.31	0.24–0.42
α -Fas	i.m.	0.04	0.02–0.07
D-Gln/LPS	i.p.	0.17	0.09–0.32
D-Gln/LPS	i.v.	0.09	0.03–0.31
D-Gln/LPS	p.o.	<0.01	

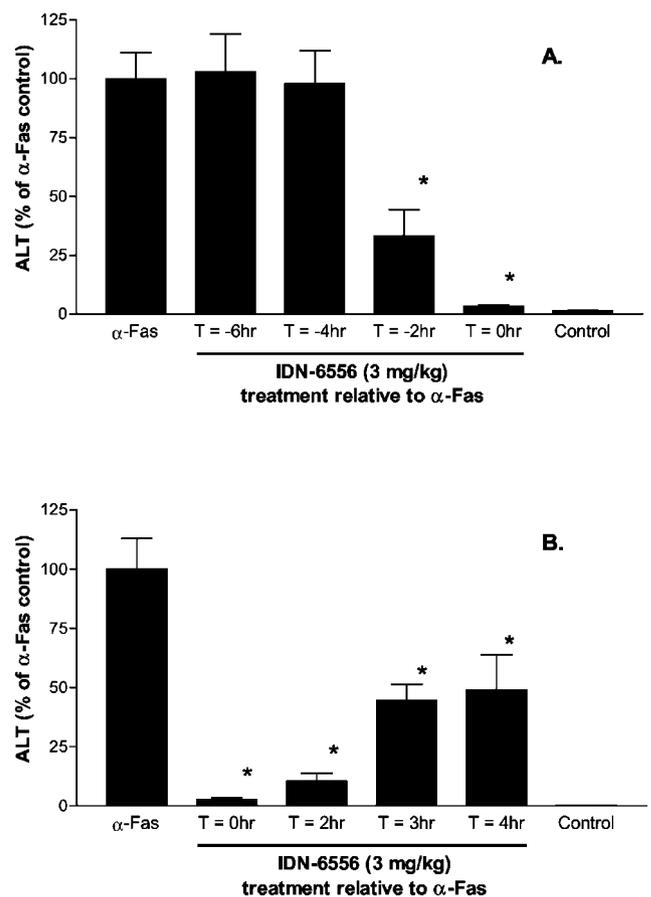


Fig. 2. Pre- and postdose efficacy by IDN-6556 in the α -Fas model. A, saline or IDN-6556 (3.0 mg/kg i.p. in saline) was administered to mice 6, 4, 2, or 0 h before α -Fas (200 μ g/kg i.v.). B, α -Fas (200 μ g/kg i.v.) was administered to mice followed by IDN-6556 (3.0 mg/kg i.p.) 0, 2, 3, or 4 h later. Mice were euthanized 6 h after α -Fas and blood was harvested. Plasma was prepared for measurement of ALT activities. ALT activities are expressed as percentage of α -Fas controls. $n = 5$ to 13/group. Asterisk (*) indicates a significant reduction from the α -Fas-treated control ($p < 0.05$).

of IDN-6556 after i.v., p.o., and i.m. administration were compared: ED_{50} values 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 postadministration of IDN-6556 was then evaluated in the α -Fas model. Preadministration of IDN-6556 (3 mg/kg i.p.) immediately or 2 h before α -Fas resulted in significant reductions in ALT activities, whereas administration of IDN-6556 4 or 6 h before did not (Fig. 2A). In contrast, drug was effective when dosed i.p. as late as 4 h post- α -Fas administration, indicating postinsult efficacy (Fig. 2B). Furthermore, in this study, 40% of mice died in the control group (4 of 10) by 24 h, whereas no mice receiving α -Fas and IDN-6556 (3 mg/kg, 0–4 h, i.p.) 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 to 8 h (Mignon et al., 1999). When IDN-6556 was given 4 h after D-Gln/LPS administration to mice and ALT activities were determined in plasma 10 h after D-Gln/LPS administration, ED₅₀ values after i.p. and i.v. 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). Furthermore, after i.p. administration, caspase activity was reduced by 74% at doses of 1 mg/kg or higher (data not shown). When drug was administered orally in two independent experiments, an ED₅₀ could not be determined because 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 47 to 76% (ED₅₀ of 1.2 mg/kg; Fig. 3). A 67% reduction in ALT activities was seen after an i.p. dose of IDN-6556 (10 mg/kg), and caspase activities were reduced by 57% with the 10 mg/kg of IDN-6556. Last, 3 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 (i.v., i.p., or s.c.), IDN-6556 had terminal half-lives of 51 ± 11, 47 ± 5, and 46 ± 8 min, respectively (Fig. 4; Table 2). Bioavailability was 49 and

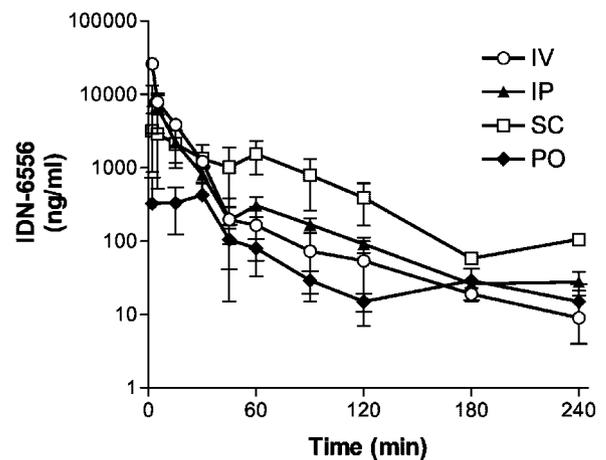


Fig. 4. Pharmacokinetic profiles of IDN-6556 after four routes of administration. Carotid artery-cannulated rats received IDN-6556 (10 mg/kg) prepared in 100 mM phosphate buffer by i.v., i.p., s.c., or p.o. administration. Blood was taken from the carotid cannula 2 to 240 min after drug administration. Plasma prepared from blood was extracted and IDN-6556 was quantified by LC/MS/MS as described under *Materials and Methods*. Data are expressed as mean ± S.D. $n = 3$ to 4/group.

70% for i.p. and s.c. 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 Fig. 4.

Because apparent oral bioavailability of IDN-6556 in the rat was low, but efficacy was similar after i.p. and p.o. administration, portal and systemic concentrations of IDN-6556 after both i.v. and p.o. administration were compared. In rats treated i.v. 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-h period, reaching 2560 ng/g liver at 120 min, and a termi-

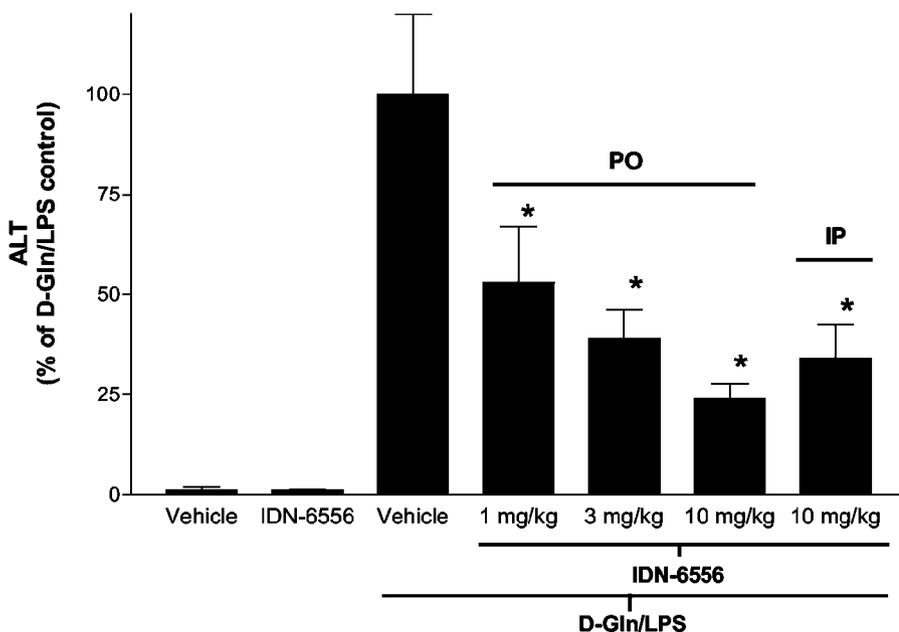


Fig. 3. Reduction of D-Gln/LPS-induced liver injury in rats by IDN-6556. Rats received 500 mg/kg D-Gln with 500 μ g/kg LPS i.p., dissolved in saline. Four hours after administration of D-Gln/LPS, rats were treated with either vehicle or drug (1, 3, and 10 mg/kg p.o. or 10 mg/kg i.p.). Ten hours after D-Gln/LPS administration, rats were sacrificed and plasma was prepared from harvested blood. ALT activities were determined in plasma and expressed as mean ± S.E.M. $n = 6$ to 8/group. Asterisk (*) indicates significant difference from D-Gln/LPS-treated control ($p < 0.05$).

TABLE 2

Pharmacokinetic parameters for IDN-6556 after various routes of administration

IDN-6556 (10 mg/kg) was administered i.v., i.p., s.c., or p.o. 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 min after administration and analyzed by LC/MS/MS as described under *Materials and Methods*. Data are expressed as mean \pm S.D. $n = 3$ to 4 rats/time point. Bioavailability was calculated from the AUC_{inf} .

Parameters	Units	i.v.	i.p.	s.c.
T_{max}	min	0	2 \pm 0	2 \pm 0
C_{max}	ng/ml	59,000 \pm 9000	8100 \pm 5200	3200 \pm 2300
$t_{1/2}$	min	51 \pm 11	47 \pm 5	46 \pm 8
AUC_{last}	ng \cdot h/ml	4200 \pm 320	2100 \pm 520	2900 \pm 1700
AUC_{inf}	ng \cdot h/ml	4300 \pm 320	2100 \pm 500	3000 \pm 1600
V_{ss}	l/kg	0.45 \pm 0.09	N/A	N/A
CL	ml/kg/min	39 \pm 3	N/A	N/A
MRT_{inf}	min	12 \pm 3	30 \pm 9	69 \pm 11
%F	%	100	49	70

N/A, not applicable.

TABLE 3

Systemic, portal vein, and liver concentration-time curves of IDN-6556 after p.o. and i.v. administration in rats

IDN-6556 prepared in 100 mM phosphate buffer was administered p.o. or i.v. to rats (10 mg/kg). Serum samples prepared from blood taken via the portal vein and inferior vena cava ranging from 2 to 240 min. IDN-6556 concentrations were quantified in sera and harvested livers by LC/MS/MS as described under *Materials and Methods*. Data are represented as mean \pm S.D. $n = 3$ for liver and $n = 6$ portal and systemic concentrations.

	T_{max}	C_{max}	$t_{1/2}$	AUC_{last}	AUC_{inf}	V_{ss}	CL _{last}	MRT_{last}	MRT_{inf}
	min	ng/ml or ng/g	min	ng \cdot h/ml or ng \cdot h/g	l/kg	ml/kg/min apparent		min	
p.o.									
Liver	120	2600	ND	8000	ND	2900	—	137	ND
Portal	20	1030	120	540	790	—	—	88	191
Systemic	10	600	32	130	130	—	—	31	36
i.v.									
Portal	0	69,000	50	4300	4300	850	39	20	22
Systemic	0	64,000	49	3500	3500	680	47	13	14

%F systemic, $AUC_{po}/AUC_{iv} \times 100\% = 4\%$; %F portal, $AUC_{po}/AUC_{iv} \times 100\% = 12\%$; ND, not determined.

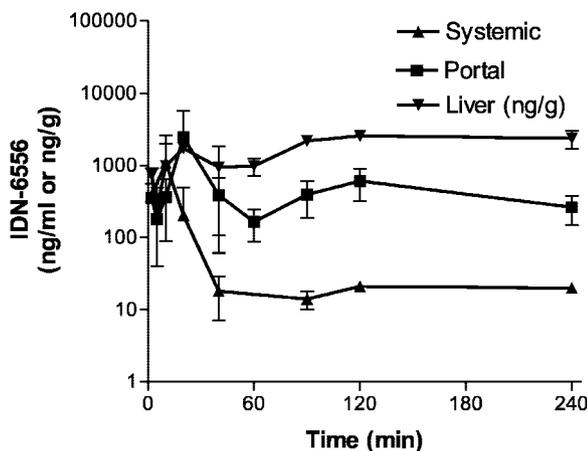


Fig. 5. Systemic, portal, and liver concentration-time curves of IDN-6556 after oral administration in rats. IDN-6556 prepared in 100 mM phosphate buffer was administered p.o. to rats (10 mg/kg). Blood was taken via the portal vein and inferior vena cava and livers were harvested 2 to 240 min after dose administration. Blood and livers were analyzed by LC/MS/MS for IDN-6556 as described under *Materials and Methods*. Data are represented as mean \pm S.D. $n = 3$ /time point.

nal $t_{1/2}$ in liver could not be determined within 4 h. IDN-6556 concentrations were greatest in the liver \gg 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 (Fig. 5).

Cumulative biliary excretion after i.v. and p.o. administration of IDN-6556 was assessed. After i.v. administration, 51 \pm 20% ($n = 4$) of an administered dose of IDN-6556 (10

TABLE 4

Microsomal and S9 stability of IDN-6556

IDN-6556 (50 μ M) or testosterone (50 μ M) were incubated with rat microsomes (active or heat-denatured) or S9 (active or heat-denatured) supplemented with UDPGA (2 mg/ml) and PAPS (100 μ g/ml) for 45 min at 37°C. The reaction was stopped with addition of acetonitrile. Compounds were extracted with acetonitrile and analyzed by high-performance liquid chromatography.

Drug	% Reduction of Drug		
	Microsomes	S9	S9 with UDPGA and PAPS
IDN-6556	4	0	5
Testosterone	85	74	56

mg/kg) was found intact in bile after 24 h with the majority excreted within 1 h. However, only 4.9 \pm 3.5% ($n = 3$) was found in bile after p.o. administration of IDN-6556 (38% of the available dose; data not shown).

Last, 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.

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 nonfunction in transplanted livers (Galle and Kramer, 1998). In these liver diseases, programmed cell death seems 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 because 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 IC₅₀ values in the low to subnanomolar range against caspases 1, 3, 6, 7, 8, and 9 as well as submicromolar efficacy in a variety of cellular assays. 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 postinsult. Furthermore, the compound seemed 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 h later. These data confirm reports using broad-spectrum caspase inhibitors in models of 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 h after α -Fas, demonstrating postinsult efficacy. Although liver injury was not totally inhibited when compound was administered this late, lethality was completely blocked. Lack of complete efficacy is not surprising after postinsult because caspase 3-like activity was already maximal by 4 h and significant injury had already occurred (Hoglen et al., 2001). The postinsult efficacy was confirmed in the D-Gln/LPS model as dosing of IDN-6556 is effective 4 h 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 postinsult in clinical situations. In contrast, preadministration resulted in protection 2 h, but not 4 h, before 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 h, but not 24 h 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 admin-

istration, the ED₅₀ of less than 0.01 mg/kg was determined in the mouse but only 1.2 mg/kg in the rat. Furthermore, although 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 are 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. (2003), 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. But, in the murine D-Gln/LPS model, the ED₅₀ of IDN-6556 was less than 0.01 mg/kg after oral administration, and the drug was most potent by this route of administration. Comparable activity was seen in the rat where efficacy after p.o. administration was similar to i.p. administered IDN-6556. Furthermore, 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 after 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 min after i.v., i.p., and s.c. 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 h before α -Fas administration. Biological activity seems to last approximately 6 to 8 h 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 i.p. 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 after i.v. and p.o. 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 p.o. administration in portal plasma compared with systemic concentrations was approximately 4- and 6-fold greater, respectively. Furthermore, 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 h, portal and liver concentrations after p.o. administration were nearly at maximal levels. This was contrary to the pharmacokinetics seen in portal and

systemic plasma after i.v. administration, where most of the drug was eliminated by 4 h.

Rapid elimination of the drug seemed to be, at least in part, due to biliary excretion. In the rat, bile contained over 50% of the intact drug after i.v. 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 contrast, rapid elimination of the drug did not seem to involve cytochrome P450-mediated metabolism, because only a small percentage of IDN-6556 was metabolized by microsomes in the rat. Additionally, significant phase II metabolism seems unlikely because 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, because exposure to organs other than the liver and gastrointestinal tract would be lower after oral administration. It also has the advantage of site-directed targeting because 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 seemed to have high enterohepatic recirculation and first pass effect as evidenced by a secondary peak in liver and plasma as well as relatively high portal levels compared with 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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