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Preclinical Profile of VX-950, a Potent, Selective, and Orally Bioavailable Inhibitor of Hepatitis C Virus NS3-4A Serine Protease

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VX-950 is a potent, selective, peptidomimetic inhibitor of the hepatitis C virus (HCV) NS3-4A serine protease, and it demonstrated excellent antiviral activity both in genotype 1b HCV replicon cells (50% inhibitory concentration [IC_{50}] = 354 nM) and in human fetal hepatocytes infected with genotype 1a HCV-positive patient sera (IC_{50} = 280 nM). VX-950 forms a covalent but reversible complex with the genotype 1a HCV NS3-4A protease in a slow-on, slow-off process with a steady-state inhibition constant (K_i*) of 7 nM. Dissociation of the covalent enzyme-inhibitor complex of VX-950 and genotype 1a HCV protease has a half-life of almost an hour. A >4-$\log_{10}$ reduction in the HCV RNA levels was observed after a 2-week incubation of replicon cells with VX-950, with no rebound of viral RNA observed after withdrawal of the inhibitor. In several animal species, VX-950 exhibits a favorable pharmacokinetic profile with high exposure in the liver. In a recently developed HCV protease mouse model, VX-950 showed excellent inhibition of HCV NS3-4A protease activity in the liver. Therefore, the overall preclinical profile of VX-950 supports its candidacy as a novel oral therapy against hepatitis C.

Hepatitis C virus (HCV) is a positive-stranded RNA virus and is a member of the *Flaviviridae* family of viruses. The HCV epidemic worldwide, affecting ~170 million people, has been widely discussed previously (24, 41). The current standard therapy for patients with chronic hepatitis C is a combination of a weekly injection of pegylated alpha interferon (IFN-α), and a twice-a-day oral administration of ribavirin (for a review, see references 28 and 35 and references therein). Both drugs are indirect antiviral agents, since they do not target a specific HCV protein or nucleic acid. A sustained viral response, which is defined as the HCV viral load in treated patients remaining undetectable for 6 months after the termination of therapy, is achieved in only half of the treated patients. The proportion of patients achieving a sustained viral response is even less in the subsets of patients with genotype 1 HCV infection or with a high viral load (4, 6, 22). The standard therapy is associated with considerable adverse effects, including depression, fatigue, and "flu-like" symptoms caused by IFN-α and hemolytic anemia caused by ribavirin. There is currently an unmet medical need for orally available, small-molecule, direct anti-HCV drugs to provide patients with hepatitis C more effective treatments with fewer side effects.

Modern structure-based drug design techniques are well suited for the task of assembling molecular scaffolds to efficiently inhibit virus-encoded enzymes, such as proteases or polymerases that are required for propagation of virus. Of the 4 viral enzymes that are essential for HCV replication or infectivity (10), NS3-4A serine protease (5, 9, 16, 17) and NS5B RNA-dependent RNA polymerase are considered the most attractive targets for new anti-HCV oral drug development. The success of human immunodeficiency virus (HIV) protease inhibitors suggests that viral proteases, such as the HCV NS3-4A protease, could be excellent targets for a structure-based drug design approach. However, efforts to discover small-molecule, orally available, potent drug candidates have been hampered by the shallow substrate-binding groove of the HCV NS3-4A serine protease. In addition, the lack of a robust small-animal model for HCV infection has generally forced scientists to rely on a combination of anti-HCV activity in cell culture and animal pharmacokinetics as surrogate indicators of efficacy before human trials. Nevertheless, significant progress has been made in recent years to identify potent small-molecule inhibitors against the HCV protease (for a review, see references 2 and 30). Clinical proof of concept for HCV NS3-4A protease inhibitors was recently obtained when BILN 2061, a noncovalent inhibitor of HCV NS3-4A protease developed by Boehringer-Ingleheim, was shown to reduce the plasma viral load in genotype 1 HCV-infected patients by as much as ~2.5 to 3.0 $\log_{10}$ after a 2-day administration with doses up to 500 mg every 12 h (7, 11).

VX-950, a novel small-molecule, peptidomimetic inhibitor of HCV NS3-4A protease, was discovered using a structure-based drug design approach. In a 14-day phase 1b trial of VX-950 in genotype 1 HCV-infected patients, a 4.4-$\log_{10}$ me-

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dian reduction in the plasma viral load was observed in a group of patients dosed with 750 mg of VX-950 every 8 h. In some patients dosed with VX-950, the virus became undetectable (<10 IU/ml) at day 14 of dosing (H. W. Reesink, S. Zeuzem, A. van Vliet, L. McNair, S. Purdy, H.-M. Chu, and P. L. M. Jansen, Abstr. 36th Annu. Digestive Dis. Wk., abstr. 527, 2005).

Here, we summarize the preclinical profile of VX-950, a reversible and tight-binding inhibitor of the HCV NS3-4A protease. VX-950 possesses excellent antiviral activity in both HCV replicon cells and human fetal hepatocytes infected with HCV-positive patient sera. In addition, VX-950 exhibits a favorable pharmacokinetic profile in several animal species and demonstrates potent anti-HCV protease activity in a mouse model for the HCV NS3-4A protease. These results are commensurate with the properties expected for a clinically viable drug.

MATERIALS AND METHODS

Materials. VX-950 (compound 2 in Fig. 1A and Table 1) was prepared at Vertex Pharmaceuticals, Incorporated, as described previously (43). The P1 *R*-diastereomer (compound 3 in Table 1) and the P1 diastereomeric mixture (compound 4 in Table 1) of VX-950 were also synthesized at Vertex Pharmaceuticals, Incorporated. The NS3 protease domain of the HCV H strain was expressed and purified as described previously (9). The NS4A cofactor peptide, based on the consensus sequences of HCV genotype 1 isolates, KK4A (NH_2-KKGSVVIVGRIVLSGK-COOH) (12), was purchased from American Peptide Company, Inc. (Sunnyvale, CA). The peptide substrate NS5A/5B (H-EDVVαAbuCSMSY-OH) and the fluorescent substrate RET-S1 (Ac-DED[EDANS]EEαAbuψ[COO]ASK[DABCYL]-NH_2) (37) were bought from AnaSpec, Inc., (San Jose, CA). The following biochemicals were purchased from Bachem (King of Prussia, PA): H-[D-Val]-Leu-Arg-pNA, Bz-Phe-Val-Arg-pNA, Z-[D-Phe]-Pro-(3-methoxypropyl)boroglycine-pinanediol ester, H-[D-Ile]-Phe-Lys-pNA, H-[D-Phe]-Pro-Arg-chloromethylketone, Boc-Ile-Glu-Gly-Arg-AMC. Leupeptin was from Sigma (St. Louis, MO). Human kallikrein and plasmin were from Calbiochem (La Jolla, CA). Human factor Xa and thrombin were from Enzyme Systems Products (Livermore, CA). HCV-positive human serum was purchased from Sacramento Blood Center (Sacramento, CA).

Fluorescence peptide cleavage assays for HCV NS3 protease. The steady-state inhibition constant (K_i^*) of VX-950 was determined in an assay that was modified slightly from a fluorescence peptide cleavage assay described by Taliani et al. (37). The assay was performed in a buffer containing 50 mM HEPES (pH 7.8), 100 mM NaCl, 20% glycerol, and 5 mM dithiothreitol (buffer A), using the RET-S1 fluorescent peptide as the substrate. Reactions were continuously monitored using an fMax fluorescence microtiter plate reader (Molecular Devices, Sunnyvale, CA) thermostatted at 30°C, with excitation and emission filters of 355 nm and 495 nm, respectively. A stock solution of HCV NS3 protease in buffer A containing 25 μM KK4A peptide was preincubated for 10 min at room temperature, followed by an additional 10 min of incubation at 30°C. An aliquot of VX-950, dissolved in 100% dimethyl sulfoxide (DMSO), was added to a solution of RET-S1 in buffer A containing 25 μM KK4A peptide and preincubated at 30°C for 10 min. The reaction was initiated by the addition of an aliquot of the NS3 protease/KK4A stock to the VX-950/RET-S1/KK4A/buffer A mixture to yield final concentrations of 12 μM RET-S1, 2% (vol/vol) DMSO, 25 μM KK4A peptide, and 0.5 to 1.0 nM HCV NS3 protease. Steady-state reaction rates were determined from linear regression of the fluorescence versus time data points obtained over a 5-min window at a reaction time of 4 h. The K_i^* of VX-950 was determined by fitting activity versus inhibitor concentration data to the Morrison equation for tight-binding enzyme inhibition (25). The dissociation rate constant of the complex between HCV NS3 protease and VX-950 was determined using the RET-S1 substrate as follows. A stock solution of HCV NS3 protease in buffer A containing 25 μM KK4A peptide was prepared as described above. A 1-μl aliquot of 100 μM VX-950 dissolved in 100% DMSO was added to a 49-μl aliquot of the prewarmed enzyme stock to yield a mixture of 320 nM enzyme and 2 μM VX-950, which was then incubated at 30°C for 4 h to allow formation of the enzyme-inhibitor complex to reach equilibrium. The dissociation reaction was initiated by serial dilution of an 8-μl aliquot of the enzyme-inhibitor mixture into 192 μl of buffer A containing 25 μM KK4A peptide and 2% DMSO (vol/vol)

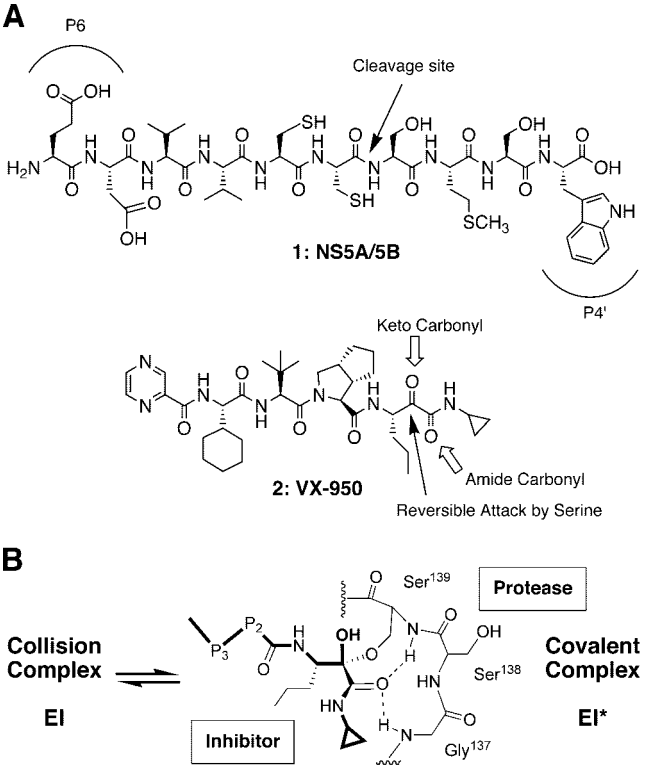
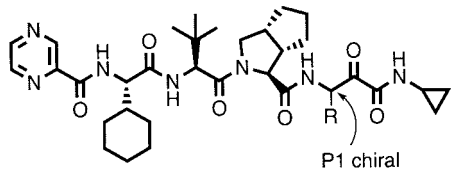


FIG. 1. (A) Chemical diagrams of the natural NS5A/5B substrate (compound 1), with its P6 to P4′ residues and the cleavage bond (indicated by solid arrow), and the HCV NS3-4A protease inhibitor VX-950 (compound 2), with the bond attacked by Ser^{139} of HCV protease (indicated by a solid arrow) and two carbonyl groups (indicated by an open arrow). (B) Schematic representation of the covalent EI* complex structure based on an X-ray co-crystal structure of the HCV NS3-4A protease and VX-950. The inhibitor is shown at the left and the protease at the right. A covalent but reversible bond is formed between the hydroxyl group of catalytic Ser^{139} of the HCV protease and the keto-carbonyl group of VX-950. In addition, two hydrogen bonds (indicated by dashed lines) are formed between two main chain amide NH groups of the protease (Gly^{137} and Ser^{139}) and the amide carbonyl group of VX-950.

and then into 192 μl of RET-S1 in buffer A containing 25 μM KK4A peptide and 2% DMSO, both prewarmed to 30°C. Final concentrations were 0.5 nM HCV NS3 protease, 25 μM KK4A peptide, 12 μM RET-S1, and 3 nM VX-950. The change in fluorescence was monitored over a 4-h window, and the fluorescence versus time data plots were fit to the following equation by nonlinear regression: $F(t) = Vs \times t + (Vi - Vs) \times [1 - \exp(-k_{obs} \times t)]/k_{obs} + C$. Control rates were determined from a reaction containing neat DMSO. Under these experimental conditions, k_{obs} is within 20% of k_{off}. The half-life of the complex ($t_{1/2}$) was calculated from k_{off} using the following equation: $t_{1/2} = 0.693/k_{off}$.

High-performance liquid chromatography-based peptide cleavage assay for HCV NS3 serine protease. This assay is a slightly modified version of what has been previously described (12). The NS3 protease (10 to 25 nM) and 25 μM KK4A were preincubated for 5 min in a buffer containing 50 mM HEPES (pH 7.8), 100 mM NaCl, 20% glycerol, and 5 mM dithiothreitol at room temperature (12). HCV protease inhibitors, dissolved in DMSO, were added to the enzyme mixture, with a final DMSO concentration of 2% (vol/vol), and incubated for 15 min at room temperature. The proteolysis reaction was initiated by the addition of NS5A/5B substrate at a concentration equal to its K_m (25 μM) and incubated for 15 min at 30°C. The reaction was quenched by the addition of a one-fourth volume of 10% trifluoroacetic acid and analyzed on a reversed-phase high-performance liquid chromatography column. Sample analysis was completed within 24 h of reaction termination. The apparent inhibition constant [$K_{i(app)}$] of HCV protease inhibitors was calculated using a least-squares fitting method of

TABLE 1. Enzyme and replicon activities of P1 diastereomers of VX-950[a]



Compound	R	P1 chiral conformation	$K_{i(app)}$ (μM)	Replicon cell assay results		
				IC_{50} (μM)	CC_{50} (μM)	SI
2 (VX-950)		*S*	0.044	0.354	82	230
3		*R*	1.190	0.883	>100	>113
4		*S* + *R*	0.078	0.459	95	206

[a] The $K_{i(app)}$ values of VX-950 (compound 2, the P1 *S*-diastereomer), compound 3 (the P1 *R*-diastereomer), and compound 4 (P1 diastereomeric mixture) were determined in an enzyme assay in which the compounds were preincubated with the purified NS3 protease domain and the KK4A cofactor peptide of the HCV H strain (genotype 1a) for 15 min and incubated with the genotype 1 NS5A/5B substrate for another 15 min. The IC_{50}s and CC_{50}s were determined in the standard 48-h Con1 (genotype 1b) subgenomic replicon cell assay. The SI (selective index) is the ratio of CC_{50} to IC_{50}.

nonlinear regression based on Morrison's equation for tight-binding competitive inhibition (25).

Selectivity assays for other serine proteases. Protease selectivity assays were conducted in a buffer containing 50 mM HEPES (pH 7.8), 100 mM NaCl, 20% glycerol, and 2% DMSO (vol/vol) (buffer B). A 2-μl aliquot of VX-950 dissolved in 100% DMSO was added to a 78-μl enzyme stock solution freshly prepared in buffer B and then preincubated for 15 min at 30°C. The reaction was initiated by the addition of 20 μl of a substrate stock solution in buffer B to obtain a final substrate concentration equal to the K_m. For substrates releasing *p*-nitroaniline (pNA), the rate of reaction was obtained by monitoring the increase in absorbance at 405 nm relative to that at 650 nm as a function of time, using a Thermomax spectrophotometer (Molecular Devices) thermostatted at 30°C. For substrates releasing 7-amino-4-methylcoumarin (AMC), the rate of reaction was obtained by monitoring the increase in fluorescence at 460 nm following excitation at 390 nm as a function of time, using an fMax fluorescence microtiter plate reader (Molecular Devices) thermostatted at 30°C. The percent inhibition was calculated relative to a control reaction in which compound was replaced with neat DMSO. Human kallikrein was assayed at a final concentration of 2.5 nM using 200 μM H-[D-Val]-Leu-Arg-pNA substrate, with leupeptin included as a positive control. Human thrombin was assayed at a final concentration of 0.2 units/ml using 350 μM Bz-Phe-Val-Arg-pNA as a substrate, with Z-[D-Phe]-Pro-(3-methoxypropyl)boroglycine-pinanediol ester included as a positive control. Human plasmin was assayed at a final concentration of 0.005 units/ml using 20 μM H-[D-Ile]-Phe-Lys-pNA as a substrate, with H-[D-Phe]-Pro-Arg-chloromethylketone included as a positive control. Human factor Xa was assayed at a final concentration of 5 nM using 600 μM Boc-Ile-Glu-Gly-Arg-AMC as a substrate, with H-[D-Phe]-Pro-Arg-chloromethylketone included as a positive control.

IC_{50} determination in HCV replicon cells. Huh-7 cells harboring an autonomously replicating, subgenomic HCV replicon of the Con1 strain (19) were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and nonessential amino acids (JRH Biosciences, Lenexa, KS), plus 0.25 mg/ml G418 (Invitrogen, Carlsbad, CA). The subgenomic HCV replicon also encodes a neomycin phosphotransferase, which allows selective growth of HCV replicon-containing Huh-7 cells over HCV replicon-negative Huh-7 cells in the presence of G418. The VX-950 concentrations at which the HCV RNA level in the replicon cells is reduced by 50% (IC_{50}) or by 90% (IC_{90}) or the cell viability is reduced by 50% (CC_{50}), were determined in HCV Con1 subgenomic replicon cells (19) using 4-parameter curve fitting (SoftMax Pro) as described previously (15, 18). Briefly, the replicon cells were incubated with compounds diluted in DMEM containing 2% FBS and 0.5% DMSO (without G418) at 37°C. Total cellular RNA was extracted using an RNeasy-96 kit (QIAGEN, Valencia, CA), and the copy number of the HCV RNA was determined in a quantitative, real-time, multiplex reverse transcription-PCR (QRT-PCR, or Taqman) assay (18). The cytotoxicity of compounds in the HCV replicon cells was measured under the same experimental settings using the tetrazolium-based cell viability assay as described before (15, 18).

Nine-day HCV replicon clearance assay. The 9-day HCV replicon clearance assay was carried out in the absence of selection pressure from G418 as described before (18). Briefly, HCV replicon cells were plated at a density of 500 cells per well in a 96-well plate and were incubated with 7 μM VX-950 or 0.2% DMSO in DMEM containing 10% FBS (without G418) for 3, 6, or 9 days. The cell culture medium was replaced every 3 days with fresh medium containing VX-950 or DMSO control. At the end of 3-, 6-, or 9-day incubation, the number of cells and the level of HCV RNA in each well was determined using the tetrazolium-based cell viability assay with a standard curve of viable cells and by the QRT-PCR assay, respectively, as described before (18). The copy number of HCV replicon RNA per cell was calculated and divided by the copy number in DMSO control cells.

HCV replicon rebound assay. In the HCV replicon rebound assay, HCV replicon cells were incubated with 17.5 μM VX-950 or 0.2% DMSO in medium containing 10% FBS for 13 days in the absence of G418 and then cultured without VX-950 or DMSO in the presence of G418 selection pressure as described before (18). Briefly, HCV replicon cells were plated in a six-well plate at a density of 2.0×10^5 cells per well and incubated with VX-950 or 0.2% DMSO control in DMEM and 10% FBS (without G418) for up to 13 days, during which the replicon cells were split every 3 to 4 days and plated into fresh medium containing VX-950 or 0.2% DMSO. After 13 days of incubation, the medium was removed and the cells were split and plated into fresh medium containing 0.25 mg/ml G418 but in the absence of VX-950 or DMSO. The cells were cultured for three more weeks in the presence of 0.25 mg/ml G418, during which cells were either split or fresh medium was added every 3 to 4 days. For all the samples taken, the number of live cells and the level of HCV RNA in each sample was determined by ViaCount assay (Guava Technologies, Hayward, CA) and by the QRT-PCR assay, respectively, and then the copy number of HCV replicon RNA per cell in each sample was calculated.

HCV infection in primary human fetal liver cells. The details of the in vitro HCV infection model using isolated primary human fetal liver cells will be described elsewhere. A brief description of the model is given here. Fetal human liver tissue samples were obtained from a nonprofit research institute, Advanced Bioscience Resources, Inc. (Alameda, CA). The 21-week fetal liver tissue samples were finely dissected and then subjected to a two-step collagenase digestion procedure. Liver cells were purified by repeated low-speed centrifugation and filtration through nylon mesh, followed by plating onto collagen-coated wells containing mitomycin C (Sigma; St. Louis, MO)-treated STO feeder cell layers (21). The cultures were maintained at a density of $\sim 3 \times 10^5$ cells/well in a fetal calf serum-free, hormonally defined medium (IM-HDM) (8). Nonattached cells were removed after 24 h, and the medium was replaced every 2 to 3 days. Five days after the original plating, 200 μl of HCV serum (RNA 898, an HCV genotype 1a serum with a titer of 9.3×10^6 gEq/ml by Chiron bDNA assay; Sacramento Blood Center, Sacramento, CA) was added to 800 μl of IM-HDM in the wells and gently mixed. After 24 h, the inoculum was removed and the cells were rinsed three times with IM-HDM. Various concentrations of VX-950 diluted in IM-HDM were added to the cell culture and incubated for 48 h. The

medium was removed and replaced in its entirety with IM-HDM containing the same concentrations of VX-950 and incubated for an additional 72 h. The cell monolayer was rinsed three times and lysed, and the total cellular RNA was extracted with the RNeasy-96 kit according to the manufacturer's instructions (QIAGEN). HCV RNA levels were determined using the QRT-PCR method as described above. The IC_{50} was calculated from the percent inhibition relative to that of the control (DMSO) wells using four-parameter logistic curve fitting.

Pharmacokinetic studies in animals. The intravenous and oral pharmacokinetics of VX-950 were evaluated in rats and dogs. A group of 3 male Sprague-Dawley rats weighing 250 to 300 g (Harlan, MD) was administered an intravenous bolus dose of 0.95 mg/kg VX-950 in a vehicle consisting of 15% ethanol, 10% dimethyl isosorbide, 35% polyethylene glycol 400, and 40% D5W (5% dextrose in water). Serial blood samples were collected in heparinized tubes before dosing and at 0.083, 0.167, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 h after dose administration. A group of 3 male beagle dogs (8 to 12 kg; Charles River, MA) was administered an intravenous bolus dose of 3.5 mg/kg VX-950 in 10% ethanol, 40% polyethylene glycol 400, and 50% D5W. Serial blood samples were collected in heparinized tubes before dosing and at 0.083, 0.167, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, and 24 h after dose administration. For oral studies in rats and dogs, VX-950 was formulated in polyvinylpyrrolidone (PVP) K-30 plus 2% sodium lauryl sulfate and then dosed as an oral gavage. A group of 3 male Sprague-Dawley rats (250 to 300 g; Harlan, MD) was dosed orally with 40 mg/kg VX-950, and a group of 4 male beagle dogs (10.9 to 12.0 kg) was administered an oral dose of 9.6 mg/kg VX-950. In both oral studies, blood samples were taken before dosing and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h after dose administration. In both intravenous and oral studies, plasma samples were obtained by centrifugation and stored at −70°C until analysis. Samples from the intravenous studies were analyzed by a chiral liquid chromatography followed by tandem mass spectrometry (LC/MS/MS) method, and samples from the oral studies were analyzed using an achiral LC/MS/MS method. Briefly, VX-950 was serially diluted into normal rat or dog plasma and used as an internal control. Plasma samples were acidified and extracted by a liquid-liquid extraction procedure using a 96-well format to isolate the analytes and internal standard from rat or dog plasma. Following reconstitution, sample extracts were separated by normal-phase chromatography on a 2- by 250-mm Hypersil CPS-1 column (Thermo-Hypersil-Keystone, Bellefonte, PA) with a mobile phase of 82:17:1 heptane/acetone/methanol. Analyte concentrations were determined by turbo ion spray LC/MS/MS in the positive-ion mode. Standard noncompartmental analysis methods, using WinNonlin Enterprise/Pro, version 4.0.1 (Pharsight Corporation, Mountain View, CA), were used to calculate pharmacokinetic parameters. The following parameters were determined: C_{max}, C_{min}, or C_{avg} (maximal, minimal, or average concentration of drug in plasma, respectively), AUC_{0-8} or $AUC_{0\text{-inf}}$ (total area under the concentration curve from 0 to 8 h or from 0 to infinity, respectively), $t_{1/2}$ (half-life of elimination), CL (total body clearance), and V_{ss} (volume of distribution at steady state).

Evaluation of liver-to-plasma ratio of VX-950 in rats. The liver-to-plasma ratio of VX-950 was evaluated in rats following oral administration of a solution of VX-950 in propylene glycol. Six groups of male Fisher rats (3 animals per group) were orally administered a nominal dose of 30 mg/kg of VX-950. One group of rats was sacrificed before dosing and at 0.5, 1, 2, 4, and 8 h after dosing. One blood sample was drawn from each animal, and plasma samples were then obtained by centrifuging the blood samples. The whole liver was removed from each animal and perfused with normal saline to remove traces of blood. After weighing, the liver was cut into small pieces and homogenized with an equal volume of water. The plasma and liver samples were stored at −70°C until analysis using the achiral LC/MS/MS method.

Mouse model for HCV NS3-4A serine protease. The details of this mouse model for the HCV NS3-4A serine protease will be described elsewhere. A brief description of this model is given here. An HCV cDNA fragment was constructed to encode an initiation Met codon, a His tag (SHHHHHHAM), the full length (631 amino acids) of the HCV NS3 protein, the full length (54 residues) of the HCV NS4A protein, and the N-terminal 6 amino acids (ASHLPY) of the HCV NS4B protein. This HCV cDNA fragment was fused to a full-length secreted placental alkaline phosphatase (SEAP) gene, using overlapping PCR from pYes2-NS3-4A plasmid (23) and pSEAP2 (Clontech, Palo Alto, CA), and then subcloned into an adenovirus expression vector, pAdenovirus (Clontech), to generate pAd-WT-HCVpro-SEAP. A corresponding version of this fusion gene with an Ala substitution of the catalytic Ser^{139} in the active triad of HCV NS3-4A serine protease (5), pAd-MT-HCVpro-SEAP, was generated by the same overlapping PCR and subcloning method using a pYes2/NS3-4A containing the Ser^{139}-to-Ala mutation (23). Adenovirus was packaged by transfection of HEK293 cells (ATCC, Rockville, MD) with PacI-linearized pAdenovirus plasmid, pAd-WT-HCVpro-SEAP or pAd-MT-HCVpro-SEAP, in the presence of Lipofectamine 2000 (Invitrogen). Recombinant adenoviruses were purified by cesium chloride density gradient centrifugation and desalted by diafiltration with Centriprep YM-50 filters (Millipore, Bedford, MA). Adenovirus rapid titer kits (Clontech) were used to determine the amount of infectious units (IFU) of recombinant adenovirus stocks. Six-week-old SCID mice (~20 g; Charles River, Wilmington, MA) were dosed by oral gavage with VX-950 or with vehicle alone. Two hours after dosing, recombinant adenovirus (Ad-WT-HCVpro-SEAP or Ad-MT-HCVpro-SEAP) was injected into the lateral tail veins of the mice. Experimental criteria prospectively stated that animals with incomplete injections would not be included in the data analysis. Mice were anesthetized with isoflurane, and blood samples were collected at different time points postinjection using retro-orbital eye bleeds or at the ultimate time point by cardiac heart puncture. Mouse serum was diluted fivefold with distilled water, and the activity of SEAP in the serum was measured using a Phospha-Light detection system (Applied Biosystems, Foster City, CA) and a Tropix TR717 microplate luminometer (Tropix, Bedford, MA). For the pharmacokinetic analysis, serial blood and liver samples were collected at 1 or 12 h after a single-dose administration from 3 mice per time point and stored at −80°C prior to analysis. The mouse liver samples were mixed with 2 volumes (vol/wt) of 2 M formic acid, homogenized, and stored at −80°C prior to analysis. The samples were analyzed using a chiral LC/MS/MS system.

RESULTS

Drug design. VX-950, compound 2, is a peptidomimetic protease inhibitor derived via structure-based drug design techniques from the sequence of the natural HCV NS5A/5B cleavage site of genotype 1, compound 1 (Fig. 1A). VX-950 incorporates an electrophilic, noncleavable C-terminal α-ketoamide group to serve as a reversible trap of the catalytic Ser^{139} of the HCV NS3-4A protease. This keto-carbonyl of the inhibitor forms a reversible covalent bond with the catalytic serine, thereby anchoring the molecular assembly, while hydrophobic side chains of the inhibitor fill several substrate-binding pockets (S1 to S4). In addition, significant binding energy is provided by formation of hydrogen bonds between backbone N-H groups of the inhibitor and the main-chain backbone of the NS3 protease domain. The details of the structure and activity relationship leading to discovery of VX-950, which includes optimization of the P3 and P4 hydrophobic groups, removal of the acidic charge at P1′, and finally, the rigidification of the P2 proline substituent into a bicyclic proline motif have been previously published (31–33, 36, 40, 42, 43). While the optimal length for recognition by the HCV NS3-4A protease is 10 amino acids in natural HCV substrates, the backbone of these inhibitors was truncated to a tetrapeptide scaffold while maintaining high binding affinity for the NS3-4A serine protease.

VX-950 forms a tight, long-lived complex with the HCV NS3-4A protease. α-Ketoamide inhibitors of thrombin have been previously shown to bind via a two-step mechanism wherein an initial inhibitor-enzyme complex is formed, which subsequently rearranges to a more stable, covalently bound hemiketal complex (13). Binding of VX-950 to the HCV NS3-4A serine protease is thought to follow a similar biphasic process. In the initial phase, a transient-collision complex is formed in which VX-950 binds weakly to the HCV protease. This is followed by a slow rearrangement to a more tightly bound, long-lived complex. This tightly bound complex is characterized by formation of a covalent bond between the serine nucleophile of the HCV protease catalytic triad and the α-ketoamide functionality (33) of VX-950 (Fig. 1B). The dissociation of this covalent complex was evaluated for the NS3 protease domain and KK4A peptide of the HCV H strain (genotype 1a). Dissociation was slow, with a half-life of 58 min.

In contrast, classical noncovalent enzyme-inhibitor complexes, as expected in the case of BILN 2061, dissociate quickly with a half-life in the order of seconds (26). The inhibition constant describing the equilibrium between the covalent tightly bound complex and free enzyme plus inhibitor (K_i^*) has been determined to be 7 nM for VX-950 with the NS3 protease domain and KK4A peptide of the HCV H strain under steady-state conditions, which is comparable to that of BILN 2061 (10 nM) in our laboratory. A similar mechanism of inhibition has been described previously for other α-ketoacid or α-ketoamide inhibitors of HCV NS3-4A protease (3, 27, 33).

Consensus amino acid sequences for the NS3 serine protease domain and KK4A peptide were derived from alignments of multiple HCV isolates for both genotype 2a and genotype 3a. These were used to develop assays analogous to those reported above for genotype 1a. Preliminary data generated with these assays indicate that VX-950 also inhibits the NS3-4A protease from genotypes 2a and 3a but with reduced potency. VX-950 binds to genotype 2a HCV NS3-4A protease with an inhibition constant of 30 to 50 nM, which is approximately four- to sevenfold higher than that of genotype 1a. The inhibition constant is ~300 nM against genotype 3a HCV NS3-4A protease, corresponding to an approximately ~40-fold reduction in potency compared with that of genotype 1a HCV protease. Nevertheless, VX-950 remains a slow-binding, slow-dissociating inhibitor of these genotype 2a and 3a proteases, with half-lives for the inhibited complexes in the range of 10 to 30 min in comparison to the half-life of nearly an hour observed for the VX-950/NS3 protease/KK4A complex of HCV H strain (genotype 1a). These results suggest that VX-950 is also a potent inhibitor of the NS3-4A proteases from genotype 2a and, to a lesser degree, genotype 3a.

Despite the covalent nature of the VX-950 inhibition of HCV protease, VX-950, when tested at 10 μM, showed no inhibition against a panel of representative serine proteases, including kallikrein, thrombin, plasmin, and factor Xa. These results indicate that VX-950 is a selective inhibitor of HCV NS3-4A serine protease.

VX-950 clears viral RNA and prevents rebound in HCV replicon cells. The antiviral activity of VX-950 was examined in HCV Con1 (genotype 1b) subgenomic replicon cells. First, the IC_{50} of VX-950 was determined in a 48-h incubation assay. Incubation of HCV replicon cells with VX-950 resulted in a concentration-dependent decline of the HCV RNA levels, as measured by the QRT-PCR (Taqman) method (Fig. 2). The average IC_{50} (±standard deviation [SD]) of VX-950 in three independent assays was 354 ± 35 nM in the 48-h assay. Cell viability assays demonstrated that VX-950 did not cause significant cytotoxicity to replicon cells at concentrations up to 50 μM (data not shown). The CC_{50} (±SD) of VX-950 in replicon cells was determined to be 83 ± 27 μM, which results in a selectivity index window, the ratio of CC_{50} over IC_{50}, of 230 ± 59. VX-950 (compound 2) has the *S* configuration at its P1 chiral center. Not unexpectedly, the P1 *R*-diastereomer of VX-950 (compound 3) is nearly 30-fold less potent in the enzyme assay against the HCV NS3-4A serine protease than VX-950 (Table 1). The P1 diastereomeric mixture (compound 4) behaved similarly to VX-950 in enzyme assay. However, when the antiviral activities of these 3 compounds were examined in the standard 48-h HCV replicon cell assay, their IC_{50}s were surprisingly similar to each other (Table 1). These results suggest that the compounds might have epimerized at the P1 position in the replicon cells over the 48-h incubation time, ending up with the same equilibrium mixture regardless of the chiral composition of the starting compounds. In contrast, the incubation time in the enzyme assay was only 30 min, insufficient time for the pure *S*- or *R*-diastereomer to epimerize into an equilibrium mixture (data not shown).

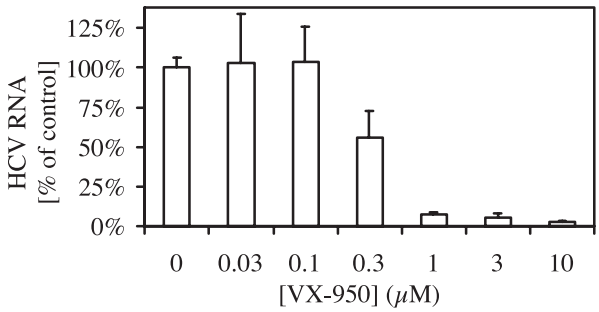


FIG. 2. Concentration-dependent reduction of HCV replicon RNA levels by VX-950. HCV replicon cells were incubated with various concentrations of VX-950 for 48 h. At the end of the 48-h incubation, total cellular RNA was extracted by RNeasy-96. The level of HCV replicon RNA remaining was then determined by QRT-PCR, as described in Materials and Methods, and was shown as a percentage of replicon RNA levels in control cells incubated with 0.5% DMSO. Each bar represents the average of results from 5 cell culture replicates with the SD. Data for one representative IC_{50} determination is shown.

The goal of anti-HCV therapy is to completely eradicate the virus and to prevent a rebound after the termination of treatment, also known as sustained viral response. To evaluate the ability of VX-950 to eradicate the viral RNA in vitro, we investigated whether multilog reduction or eventual clearance of HCV RNA from the replicon cells could be achieved upon incubation with VX-950. Replicon cells were incubated with 7 μM VX-950 for an extended period of up to 9 consecutive days. The HCV replicon RNA levels in the cells (measured by the QRT-PCR assay) were normalized to the number of viable cells (determined by the MTS cell viability assay) to preclude potential cytotoxic or cytostatic effects of the inhibitor. As shown in Fig. 3, a time-dependent decline in HCV replicon RNA levels resulted in a nearly 4-log_{10} reduction after the 9-day incubation with 7 μM VX-950, with minimal cytotoxicity observed (data not shown). We then wanted to confirm that viable HCV replicon RNA was eliminated from the cells after an extended incubation with VX-950. To identify any remaining cells harboring the HCV replicon RNA construct, which also encoded neomycin phosphotransferase conferring neomycin resistance, G418 was added back at the end of the VX-950 incubation period to allow for selective growth (defined as "rebound") of any remaining replicon-containing cells but not the "cured" Huh-7 cells, in which no replication-competent HCV RNA remains. The replicon cells were incubated with 17.5 μM VX-950 for 13 days in the absence of G418, subcultured every 3 to 4 days, and plated into fresh medium with 17.5 μM VX-950. Again, the HCV replicon RNA levels in the cells (measured by the QRT-PCR assay) were normalized to the number of viable cells (determined by the ViaCount cell via-

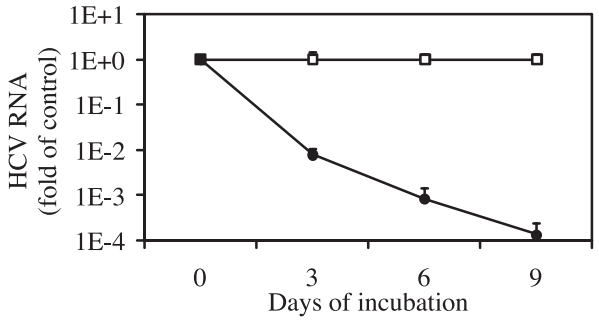


FIG. 3. Nine-day HCV replicon clearance assay with VX-950. The cells were incubated with 0.2% DMSO (open square) or 7 μM VX-950 (filled circle) for 3, 6, or 9 days. Fresh 0.2% DMSO or 7 μM VX-950 in medium was added to the cells every 3 days. At the end of each incubation period, cell numbers were determined by the tetrazolium-based cell viability assay based on an established standard curve, and the level of HCV RNA in the cells was determined by the QRT-PCR assay. The copy number of HCV RNA per cell in each sample is plotted as relative (on a log scale) to that in replicon cells incubated with 0.2% DMSO (control) for the same period of time. Each datum point represents the average of the results from 5 cell culture replicates with the SD.

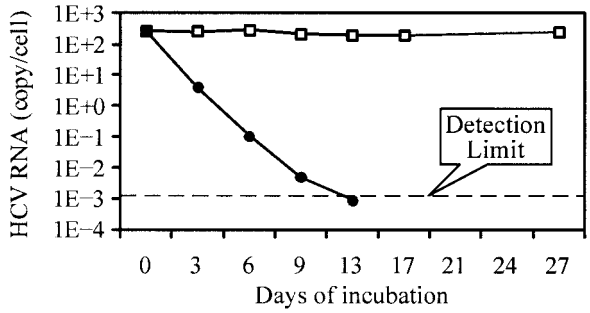


FIG. 4. Lack of rebound of HCV replicon RNA after a 13-day incubation with VX-950. HCV replicon cells were incubated with 0.2% DMSO (open square) or 17.5 μM VX-950 (filled circle) in the absence of G418. Cells were split every 3 to 4 days, and fresh 0.2% DMSO or 17.5 μM VX-950 in medium was added to the cells. After 13 days of incubation, VX-950 was withdrawn and 0.25 mg/ml G418 was added to enrich the remaining HCV replicon-positive cells that are capable of growing in the presence of G418 (rebound). The cultures were monitored for another 14 days in the presence of G418. The cell number was determined by the Guava ViaCount assay, as described in Materials and Methods, and the level of HCV RNA in the cells was determined by the QRT-PCR assay. The absolute numbers of HCV replicon RNA copies per viable cell are shown. The detection limit of the QRT-PCR assay is indicated by a dashed line.

bility assay) to preclude potential cytotoxic or cytostatic effects of the inhibitor. In control cells incubated with 0.2% DMSO, HCV RNA levels remained stable for 13 days in the absence of G418. In cells incubated with VX-950, HCV RNA levels were reduced by >4 $\log_{10}$ and dropped below the limit of detection of the QRT-PCR assay (Fig. 4). At the end of the 13-day incubation, VX-950 was removed and G418 was then added to kill the "cured" Huh-7 cells and allow growth of any cells containing replication-competent HCV replicon RNA. After 3 weeks of culture in the presence of G418, no viable replicon cells were recovered in the culture that was originally incubated with VX-950 (Fig. 4), indicating that a 13-day incubation with VX-950 was able to effectively clear viable HCV replicon RNA from Huh-7 cells under these conditions.

Inhibition of HCV replication in primary human fetal liver cells by VX-950. Few reports are available concerning in vitro infection systems with human liver cells due to the difficulty in obtaining appropriate starting tissue and uncertainty about appropriate culture conditions (1). To address these limitations, we established an in vitro infection system using primary human fetal liver cells and hormonally defined media. These primary human fetal hepatocytes remained viable for up to 28 days. Infection with many HCV sera, including samples of genotypes 1a, 1b, 2, and 3, were unsuccessful in initiating significant HCV replication, with the HCV RNA levels steadily declining as the inoculated cultures were washed and maintained (data not shown). A reproducible infection by selected HCV sera was demonstrated. The level of infection in the control wells of this experiment was approximately 1×10^4 HCV RNA copies per well, which is more than 100 times the limit of detection for HCV RNA in our QRT-PCR system. As shown in Fig. 5, VX-950 demonstrated a concentration-dependent reduction of HCV RNA levels in the human fetal liver cell cultures that were infected with an HCV patient serum of genotype 1a. No HCV RNA was detected in control wells not receiving the HCV serum. Microscopic observation of the cultures did not indicate gross changes in morphology of cells due to either infection or incubation with VX-950. The concentration-response relationship, analyzed using four-parameter curve fitting, yielded an IC_{50} of 280 nM. This value for an HCV genotype 1a serum is similar to that obtained with the HCV genotype 1b replicon system (354 nM).

Pharmacokinetics of VX-950. All the pharmacokinetic parameters in this report are presented as average values ± SD. The summary of pharmacokinetic parameters for VX-950 in rats and dogs following intravenous administration are shown

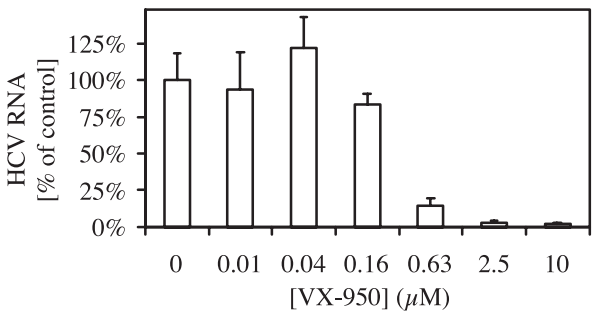


FIG. 5. Concentration-dependent reduction of HCV RNA in infected primary fetal hepatocytes by VX-950. The primary human fetal hepatocytes were isolated from a fetal liver and plated as described in Materials and Methods. Serum from an HCV-infected patient was used to infect the primary fetal hepatocytes 5 days after the plating. The inoculum was removed 24 h later, and the cells were incubated with VX-950 for 5 days. At the end of the 5-day incubation, the cells were washed and total cellular RNA was extracted by RNeasy-96. The level of HCV replicon RNA remaining was then determined by QRT-PCR, as described in Materials and Methods, and was shown as a percentage of the level of replicon RNA in cells incubated with 0.5% DMSO (control). Each bar represents the average of the results from 4 to 6 cell culture replicates with the SD.

TABLE 2. Pharmacokinetic parameters of intravenously administered VX-950 in rats and dogs[a]

Species	Dose (mg/kg of body wt)	AUC_{0-inf} (μg · h/ml)	C_{max} (μg/ml)	CL (ml · min^{-1} · kg^{-1})	$t_{1/2}$ (h)	V_{ss} (liters/kg)
Rat	0.95	0.30 ± 0.02	0.51 ± 0.06	53.7 ± 4.2	1.73 ± 0.06	5.81 ± 1.13
Dog	3.5	1.47 ± 0.44	2.38 ± 0.82	41.8 ± 10.7	0.93 ± 0.14	1.84 ± 0.44

[a] VX-950 was dosed intravenously as a solution at the indicated dose in Sprague-Dawley rats or beagle dogs. The averages ± SD of results from 3 animals are shown.

in Table 2. The plasma samples were analyzed using the chiral LC/MS/MS methods so that the intravenous pharmacokinetic values for the P1 *S*-diastereomer, VX-950 itself, are reported here. In rats, VX-950 had a $t_{1/2}$ (average ± SD) of 1.73 ± 0.06 h, a systemic clearance of 53.7 ± 4.2 ml min^{-1} kg^{-1}, and a V_{ss} of 5.81 ± 1.13 liter/kg. In dogs, VX-950 had a $t_{1/2}$ of 0.93 ± 0.14 h, a systemic clearance of 41.8 ± 10.7 ml min^{-1} kg^{-1}, and a V_{ss} of 1.84 ± 0.44 liter/kg. In rats, VX-950 exhibited a moderate-to-high systemic clearance and a V_{ss} much greater than the total body water, suggesting good distribution into the tissues. In dogs, VX-950 showed a moderate-to-high systemic clearance and a V_{ss} greater than the total body water, suggesting a moderate-to-high distribution into the tissues.

Oral administration with an amorphous suspension of VX-950 in a PVP polymer matrix resulted in good exposure in rats and dogs (Table 3). The oral pharmacokinetic studies were conducted using an achiral LC/MS/MS separation method so that the parameters of the achiral mixture of VX-950 and the P1 *R*-diastereomer, compound 3, are reported for all oral studies. At a dose of 40 mg/kg, VX-950 achieved a C_{max} of 1.55 ± 0.66 μg/ml and an AUC_{0-inf} of 3.34 ± 0.35 μg · h/ml, which resulted in an oral bioavailability of 25.0 ± 2.55% in rats. At a dose of 9.6 mg/kg, VX-950 had a C_{max} of 1.08 ± 0.45 μg/ml and an AUC_{0-inf} of 1.64 ± 0.89 μg · h/ml, which corresponded to an oral bioavailability of 40.7 ± 22.1% in dogs. The terminal $t_{1/2}$ of VX-950 following oral administration was determined to be 3.32 ± 1.71 h in rats and 3.14 ± 2.46 h in dogs.

Hepatitis C is a liver disease, in which the majority of viral infection and replication occurs in the liver. Therefore, it is a crucial feature for potential anti-HCV drugs to achieve adequate exposure in the liver. As shown in Table 4, rats were orally administered a single dose of VX-950 at 30 mg/kg in propylene glycol, and the concentrations of VX-950 in both liver and plasma were determined by the achiral method. Significantly higher concentrations of VX-950 were achieved in the liver relative to that in plasma at all time points tested (0.5, 1, 2, 4, and 8 h). The average (0 to 8 h) liver concentration in rats was 9.82 ± 5.00 μg/g versus the average plasma concentration of 0.28 ± 0.19 μg/ml, resulting in a liver-to-plasma ratio of 35 to 1, assuming the density of the liver is 1.0 g/ml.

VX-950 inhibits HCV NS3-4A serine protease in a mouse model. Given the lack of a robust and reproducible small-animal model for HCV infection, we developed a surrogate mouse model in which the activity of HCV NS3-4A serine protease expressed in mouse liver was measured by the activity of a reporter gene product. This model relied on the use of an adenovirus delivery of an inserted gene, Ad-WT-HCVpro-SEAP, containing a fused SEAP, which is released into the blood by active HCV protease. When injected into the mouse tail vein, the recombinant adenovirus concentrates in the liver, where it induces robust expression of its encoded proteins. Two groups of SCID mice (6 animals per group) were injected with 5×10^9 IFU per mouse of recombinant adenovirus, Ad-WT-HCVpro-SEAP or Ad-MT-HCVpro-SEAP, respectively, via the tail vein. At 24 h postinjection, the average serum SEAP activity (±standard error of the mean [SEM]) in the group of 6 mice injected with Ad-WT-HCVpro-SEAP was $5.12 \times 10^7 \pm 1.16 \times 10^7$ relative light units (RLU)/ml of serum, which was ~55-fold higher than the SEAP activity in the group injected with Ad-MT-HCVpro-SEAP ($9.32 \times 10^5 \pm 0.77 \times 10^5$ RLU/ml of serum) and ~70-fold higher than that in the control group with no injection ($7.30 \times 10^5 \pm 0.93 \times 10^5$ RLU/ml of serum) (Fig. 6A). These results indicate that the elevated serum SEAP activity is correlated with activity of HCV NS3-4A serine protease, which cleaves the WT-HCVpro-SEAP fusion protein and allows subsequent secretion of SEAP protein into the blood circulation. Since the vast majority of adenovirus infection and protein expression occurs in the liver after tail vein injection, the level of SEAP activity in serum can be used as a surrogate readout for the activity of HCV NS3-4A serine protease expressed in mouse liver.

Next, we tested the ability of VX-950 to inhibit HCV protease-dependent cleavage and subsequent secretion of SEAP from the liver into the blood in these mice. Five groups of 6-week-old SCID mice (6 animals per group) were injected with 10^9 IFU per mouse of recombinant adenovirus Ad-WT-HCVpro-SEAP through the tail vein. Each group of mice was given two oral administrations of VX-950 at one of the following doses: 10, 25, 75, 150, or 300 mg/kg. The first VX-950 dose was given 2 h before the adenovirus injection, and the second dose was given 10 h after injection. An additional group of 10 mice was given vehicle alone. Serum samples were collected 24 h postinjection, and the SEAP activity in each VX-950-dosed group was compared to that of the vehicle group. As shown in Fig. 6B, there was an ~5-fold reduction of serum SEAP activity in mice dosed with VX-950 at either 10 or 25

TABLE 3. Pharmacokinetic parameters for orally administered VX-950 in rats and dogs[a]

Species	Dose (mg/kg)	AUC_{0-inf} (μg · h/ml)	C_{max} (μg/ml)	T_{max} (h)	$t_{1/2}$ (h)	Bioavailability (%)
Rat	40	3.34 ± 0.35	1.55 ± 0.66	0.42 ± 0.14	3.32 ± 1.71	25.0 ± 2.55
Dog	9.6	1.64 ± 0.89	1.08 ± 0.45	0.44 ± 0.13	3.14 ± 2.46	40.7 ± 22.1

[a] VX-950 was dosed orally as a solution in PVP K30 plus 2% sodium lauryl sulfate plus water at the indicated dose in Sprague-Dawley rats and beagle dogs. The average ± SD values of results from 3 rats or 4 dogs are shown.

TABLE 4. Systemic plasma and liver exposure of orally administered VX-950 in rats[a]

Organ	AUC_{0-8} (μg · h/ml)	C_{max} (μg/ml)	C_{min} (μg/ml)[c]	C_{avg} (μg/ml)[c]
Liver[b]	78.5 ± 40.0	19.9 ± 10.5	3.30 ± 2.32	9.82 ± 5.00
Plasma	2.23 ± 1.53	0.49 ± 0.24	0.04 ± 0.03	0.28 ± 0.19
Liver/plasma ratio	35.1	40.6	82.5	35.1

[a] VX-950 was orally dosed as a solution in propylene glycol at 30 mg/kg in Fisher rats. The average ± SD values of results from 3 animals are shown.
[b] It is assumed that density of liver tissue is 1 g/mL.
[c] C_{min} was determined 8 h after the oral administration, and C_{avg} is for 0 to 8 h after the oral administration.

mg/kg, which had an average value (±SEM) of 18.7 ± 8.3% or 18.4 ± 5.4%, respectively, compared to those administered vehicle (100 ± 28%). These data demonstrated that VX-950 was able to inhibit the HCV NS3-4A serine protease activity in mouse liver and block cleavage and subsequent secretion of SEAP into blood circulation in these mice. Higher doses of VX-950 did not result in further reduction of SEAP levels (data not shown).

To correlate the pharmacokinetic and pharmacodynamic effect of VX-950 in the mouse model, the concentrations of VX-950 in liver and plasma were measured at 1 h and 12 h after a single oral dose of 10, 25, 50, 75, 150, or 300 mg/kg of VX-950. The liver and plasma samples were harvested and subjected to the chiral LC/MS/MS analysis. As shown in Fig. 6C, there was a dose-dependent increase in VX-950 exposure at 1 h postdosing in both liver and plasma. At the lowest dose tested (10 mg/kg), the level of VX-950 in the mouse liver 1 h after oral dosing was 3.86 ± 0.18 μg/g. Assuming the density of liver to be 1 g/ml, this liver concentration of VX-950 at 1 h corresponded to 5.68 ± 0.27 μM, which was about sixfold higher than that in the plasma (0.94 ± 0.09 μM). This liver concentration at 1 h is about 16-fold higher than the 48-h IC_{50} of VX-950 (354 nM) in HCV replicon cells. At doses higher than 10 mg/kg, the ratio of liver-to-plasma exposure of VX-950 1 h after dosing ranged from 11- to 16-fold. These results are consistent with the liver-concentrated exposure of VX-950 observed in rats. By 12 h, the level of VX-950 in the mouse liver and plasma were much lower than that at 1 h, although still measurable in the liver.

DISCUSSION

The current standard of care for chronic hepatitis C, pegylated IFN-α in combination with ribavirin, has limited efficacy, in particular against genotype 1 HCV-infected patients, who account for the majority of chronic hepatitis C patients in developed countries. Pegylated IFN-α, administered via a weekly injection, causes numerous serious side effects, such as depression, fatigue, "flu-like" symptoms, neutropenia, and thrombocytopenia. The addition of ribavirin, while enhancing the sustained viral response, causes additional side effects, such as cough, rash and pruritus, insomnia, anorexia, and, in particular, hemolytic anemia, a serious adverse effect. These side effects are sometimes dose-limiting or cause discontinuation of treatment, which further reduces the effectiveness of the combination therapy. There is an unmet medical need for novel,

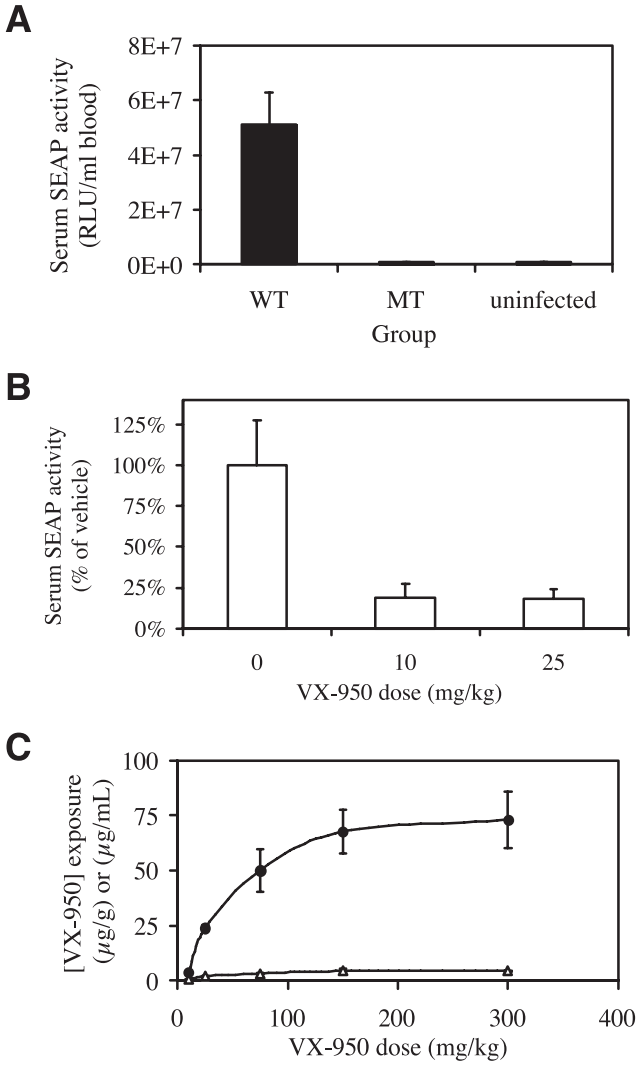


FIG. 6. VX-950 inhibits HCV NS3-4A protease and reduces SEAP levels in the NS3-4A protease mouse model. (A) Three groups of SCID mice (n = 6 per group) were given an injection of recombinant adenovirus Ad-WT-HCVpro-SEAP (WT) or Ad-MT-HCVpro-SEAP (MT) or not given an adenovirus injection (uninfected) through the tail vein. Serum collected at 24 h after injection was diluted fivefold with water, and SEAP activity was determined using the Phospha-Light detection system. Each datum point represents the average (±SEM) of SEAP activity per ml of undiluted serum in each dosing group of 6 mice. (B) Three groups of SCID mice were injected with recombinant adenovirus Ad-WT-HCVpro-SEAP through the tail vein. Two of these groups (n = 6 per group) were given 2 oral doses of VX-950 (10 or 25 mg/kg per administration): the first was given 2 h before the virus injection and the second was given 10 h after injection. The third group (n = 10) was dosed with vehicle only. Serum collected at 24 h postinjection was diluted, and the SEAP activity was determined as described above. The average (±SEM) SEAP activity per ml of undiluted serum in each dose group is plotted as the percentage of that of the vehicle group. (C) Six groups of SCID mice (n = 6 per group) were given a single oral administration of vehicle (0) or VX-950 (10, 25, 75, 150, or 300 mg/kg). The serum and liver samples were collected 1 h postdosing (shown here) from half of each dose group (n = 3) or 12 h postdosing (not shown) from the other half of each dose group (n = 3). The average VX-950 concentration at 1 h postinjection, determined by the chiral LC/MS/MS separation method, is plotted for the serum (open triangle, in μg/ml) or the liver (filled circle, in μg/g) along with SD.

safer, and more effective oral drugs, such as direct antivirals that target essential HCV-encoded enzymes.

In this report, we describe the preclinical profile of a novel inhibitor of the HCV NS3-4A protease, VX-950, which recently entered clinical development for hepatitis C. While VX-950 and BILN 2061 inhibit the genotype 1a HCV NS3-4A protease with a similar potency (steady-state inhibition constant) in enzyme assays, VX-950 has a different mode of binding than BILN 2061. BILN 2061 is a noncovalent, reversible serine protease inhibitor (11) for which the enzyme-inhibitor complex forms (fast-on) and dissociates (fast-off) quickly, presumably on the order of seconds. Conversely, VX-950 forms a covalent but reversible complex with the HCV NS3-4A serine protease in a slow process (slow-on), presumably due to the requirement of a conformational change of the initial collision complex. This allows formation of the covalent bond between the HCV protease and VX-950. Once formed, the covalent complex dissociates slowly back to free enzyme and inhibitor (slow-off). It has been determined that the half-life of the covalent complex is about 1 h. Slow-off inhibitors, such as VX-950, may offer an advantage over fast-off inhibitors for suppressing HCV RNA replication and eliminating viral RNA. The replication of viral RNA, such as HCV, occurs in a series of tightly regulated events. The viral protease, such as the HCV NS3-4A serine protease, is necessary for the processing of a polyprotein into functional, individual viral proteins that are assembled into a viral RNA replication complex. The replication of positive- and negative-stranded viral RNA is mediated by this replication complex and is highly regulated in a temporal and spatial order. A long-lasting protease-inhibitor complex could prevent the formation of a functional replication complex for a long period of time and result in a severe disruption of the highly regulated, dynamic process of viral RNA replication. In addition, the viral RNA, when prevented from being used as a template, could become more vulnerable to degradation by cellular nucleases.

In a phase 1b trial, BILN 2061 was given twice daily at 1,000 mg/day for 2 days to 8 patients infected with genotype 2 or 3 HCV. Four of 8 patients have no or weak (<1 $\log_{10}$) reduction in viral load. The antiviral activity of BILN 2061 in these patients is less pronounced and more variable than what was observed in genotype 1 HCV-infected patients (34). These results in patients are consistent with the enzyme data that BILN 2061 is a much less (~60-fold) potent inhibitor of the NS3-4A protease of genotype 2 or 3 HCV than the genotype 1 HCV protease (38). In this report, we demonstrated that VX-950 remains a potent inhibitor of genotype 2 HCV proteases in enzyme assays, with a half-life of the enzyme-inhibitor complex in the range of 10 to 30 min. These results suggest that VX-950 could be tested as a potential antiviral agent for patients infected with genotype 2 HCV.

One of the major factors limiting the efficacy of virus-specific therapies against many retroviruses and RNA viruses is the development of resistance to antiviral drugs. Resistance to many antiviral drugs, including HIV protease inhibitors, HIV or hepatitis B virus reverse transcriptase inhibitors, or influenza neuraminidase, is caused by specific mutations in the viral enzymes. In vitro resistance mutations against HCV protease inhibitors have been identified using replicon cells (14, 15, 20, 39). The major in vitro resistance mutation against VX-950 is a substitution of Ala^{156} of the NS3 protease with Ser (A156S), which remained sensitive to BILN 2061 (15). Reciprocally, the dominant in vitro resistance mutations against BILN 2061, substitutions of Asp^{168} with Ala (D168A) or Val (D168V), conferred several-hundredfold-reduced sensitivity to BILN 2061 while remaining fully sensitive to VX-950 (15). Not surprisingly, none of these 3 mutations appeared under the selective pressure of both inhibitors. Instead, substitutions of Ala^{156} by either Val (A156V) or Thr (A156T) emerged to confer cross-resistance to both VX-950 and BILN 2061 (14). It should be noted that many, if not all, of these in vitro resistance mutants remain susceptible to IFN-α and/or ribavirin and have significantly decreased replication capacity or fitness in viral RNA replication in cell culture (14, 20). It remains to be seen whether and, if so, which, resistance mutation(s), if any, will appear in HCV patients treated with new antiviral drugs. In this study, we demonstrated that a prolonged incubation with VX-950 resulted in a >4-$\log_{10}$ reduction in the HCV RNA levels in the replicon cells and no HCV replicon-containing cells remained after selection with G418 upon the withdrawal of VX-950. However, the replication rate of HCV in patients has been reported to be in the range of 10^{10} to 10^{12} viral particles per day, which is higher than the viral replication rate in HIV-infected patients (29). Further studies are needed to determine whether a single antiviral agent, such as VX-950, or a combination of two or more antiviral drugs will be needed (as in the case of HIV) to suppress the emergence of resistant variants in patients with hepatitis C.

The pharmacokinetics of peptides and peptidomimetic compounds are often problematic owing to their high molecular weights and low water solubility. VX-950 has a relatively high systemic clearance after intravenous dosing in rats and dogs. The high lipophilicity of VX-950, with a cLog *P* value of 5.2, could be advantageous for the treatment of liver disease, since first pass uptake of the drug following oral ingestion concentrates the drug in the liver, which is the primary site of HCV infection, as demonstrated in our pharmacokinetic studies. The average liver concentration (0 to 8 h) of VX-950 was 35 times the plasma concentration in rats dosed with VX-950 in propylene glycol. Since the ratio of liver-to-plasma concentrations of any given compound is usually constant regardless of vehicle, an average liver concentration (0 to 8 h) of 14 μg/g or ~20 μM is expected based on the average plasma concentration observed after an oral administration of VX-950 at 40 mg/kg in the PVP-based vehicle. The expected liver exposure in rats, ~20 μM, would be more than 50-fold the IC_{50} of VX-950 (354 nM) in the 48-h replicon cell assay. It was also reported that BILN 2061 achieved higher exposure in liver than in peripheral blood in rats after a single dose of 5 mg/kg (11).

The anti-HCV protease activity of VX-950 was also evaluated in our mouse model, in which HCV NS3-4A serine protease is expressed by adenovirus in mouse liver and its activity is measured using a surrogate readout, SEAP activity in serum. In this mouse model, VX-950 demonstrated potent inhibition against the HCV NS3-4A serine protease after two oral doses. Pharmacokinetic analysis showed that VX-950 had a dose-dependent, liver-targeting exposure in mice, similar to that observed in rats. These results are consistent with the concept of liver targeting for new anti-HCV drug candidates, such as VX-950.

In conclusion, VX-950 is a potent and selective inhibitor of the HCV NS3-4A protease in vitro with significant antiviral activity against HCV replicon cells as well as HCV infection in the primary fetal human hepatocytes. The compound exhibits good oral bioavailability in rats and dogs. In mice and rats, VX-950 achieved excellent exposure in the liver, which is the primary organ of HCV infection, suggesting that adequate exposures of the drug in humans can be achieved. Finally, VX-950 demonstrated excellent efficacy in a mouse model that we developed for the HCV NS3-4A serine protease.

Overall, the favorable preclinical data of VX-950 indicate that the compound possesses excellent biological and physicochemical properties that support further clinical development. The excellent antiviral activity observed in the recently completed phase 1b trial of VX-950 in chronic genotype 1 HCV-infected patients suggests that VX-950 has the potential to become a novel treatment option for hepatitis C and that further evaluation in advanced trials is warranted.

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