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Binding kinetics, potency, and selectivity of the hepatitis C virus NS3 protease inhibitors GS-9256 and vedroprevir

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ABSTRACT

Background: GS-9256 and vedroprevir are inhibitors of the hepatitis C virus NS3 protease enzyme, an important drug target. The potency, selectivity, and binding kinetics of the two compounds were determined using in vitro biochemical assays.
Methods: Potency of the compounds against NS3 protease and selectivity against a panel of mammalian proteases were determined through steady-state enzyme kinetics. Binding kinetics were determined using stopped-flow techniques. Dissociation rates were measured using dilution methods.
Results: GS-9256 and vedroprevir had measured K_i values of 89 pM and 410 pM, respectively, against genotype 1b NS3 protease; K_i values were higher against genotype 2a (2.8 nM and 39 nM) and genotype 3 proteases (104 nM and 319 nM) for GS-9256 and vedroprevir, respectively. Selectivity of GS-9256 and vedroprevir was >10,000-fold against all tested off-target proteases. Association rate constants of $4 \times 10^5\ M^{-1}\ s^{-1}$ and $1 \times 10^6\ M^{-1}\ s^{-1}$, respectively, were measured, and dissociation rate constants of $4.8 \times 10^{-5}\ s^{-1}$ and $2.6 \times 10^{-4}\ s^{-1}$ were determined.
Conclusions: GS-9256 and vedroprevir are potent inhibitors of NS3 protease with high selectivity against off-target proteases. They have rapid association kinetics and slow dissociation kinetics.
General Significance: The NS3 protease is a key drug target for the treatment of hepatitis C. The potency, selectivity, and binding kinetics of GS-9256 and vedroprevir constitute a biochemical profile that supports the evaluation of these compounds in combination with other direct-acting antivirals in clinical trials for hepatitis C.

1. Introduction

Over 150 million people worldwide are chronically infected with hepatitis C virus (HCV). Until recently, the standard of care for HCV patients has been a combination of pegylated interferon and ribavirin given over a period of 24–48 weeks. This regimen is not well tolerated, and the sustained viral response for this treatment is slightly above 50%. Over the last several years, many groups have targeted viral proteins for inhibitor design. One of the targets that has received a great deal of attention is the NS3 protease, a serine protease that is responsible for the cleavage of the HCV polyprotein to generate the protein components of the viral replication complex. Compounds targeting the NS3 protease include telaprevir (Incivek®), boceprevir (Victrelis®), and simeprevir (Olysio™). Telaprevir and boceprevir are peptidomimetic inhibitors with ketoamide moieties that form reversible covalent adducts with the active-site serine of NS3. Simeprevir is a product-like, non-covalent, macrocyclic inhibitor. Each of the three inhibitors is indicated for use in combination with the interferon/ribavirin standard of care. An ideal profile for NS3 protease inhibitors would include limited side effects, a low potential for drug–drug interactions, and the capacity for co-formulation with other direct-acting antivirals.

The compounds GS-9256 and vedroprevir (GS-9451) are peptidomimetic inhibitors of NS3 protease in clinical trials for HCV; their structures are shown in Fig. 1. The synthesis and antiviral properties of these compounds are reported elsewhere [1–3]. Herein we report the enzymatic characterization of GS-9256 and vedroprevir. The binding affinities of these compounds were established both by steady-state kinetics and by the independent measure of association and dissociation kinetics. The binding kinetics of GS-9256 and

Abbreviations: CatD, cathepsin D; CatL, cathepsin L; HCV, hepatitis C virus; HLE, human leukocyte elastase; PPE, porcine pancreatic elastase; Pr3, proteinase 3

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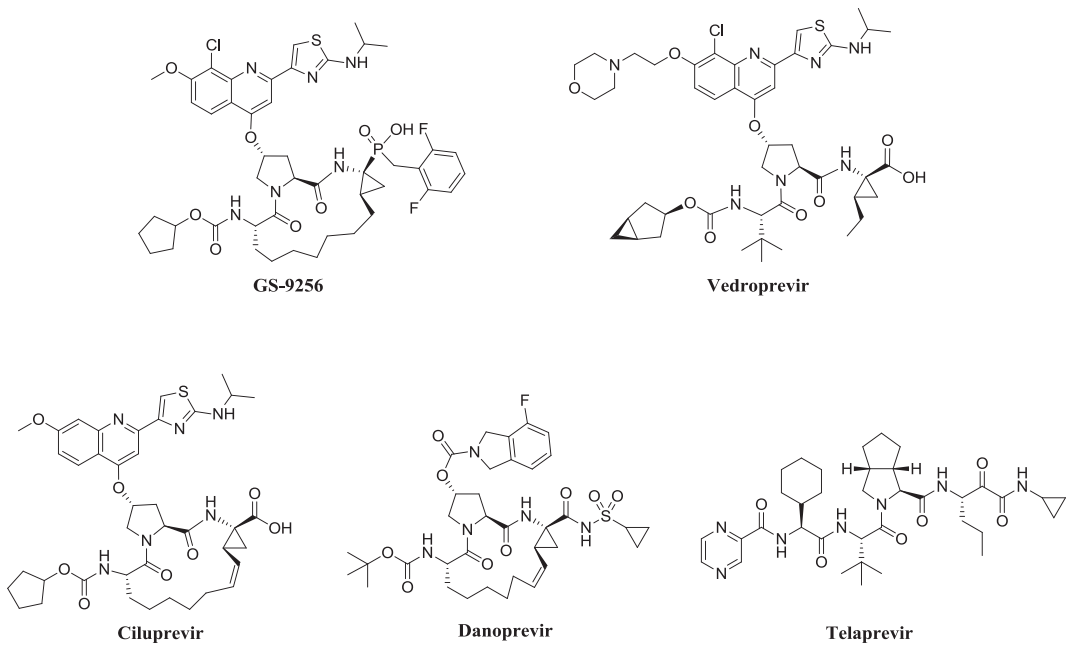

Fig. 1. Structures of inhibitors tested in this study.

vedroprevir were compared with other classes of NS3 protease inhibitors, and the selectivity of the two compounds against potential off-target mammalian proteases was determined. The enzymatic profiles of GS-9256 and vedroprevir highlight the utility of these compounds as potent and selective anti-HCV agents.

2. Materials and methods

2.1. Compounds and reagents

The synthesis of GS-9256 and vedroprevir has been described [1,3]. GS-9256, vedroprevir, and ciluprevir (BILN-2061) were manufactured by Gilead Sciences (Foster City, CA). Telaprevir (VX-950) and danoprevir (ITMN-191) were purchased from Acme Bioscience (Belmont, CA).

The internally quenched fluorogenic depsipeptide substrate Ac-DED(Edans)-EEAbuΨ[COO]ASK(Dabcyl)-NH_2 and a synthetic peptide containing the hydrophobic core residues of the NS4A protein cofactor (KKGSVVIVGRIILSGRKK; NS4A peptide) were obtained from Anaspec, Inc. (San Jose, CA). Other chemicals and biochemicals were of reagent grade or better and were purchased from standard suppliers.

Unless otherwise stated, enzymatic reactions were run in a buffer consisting of 50 mM HEPES, 40% glycerol, 0.05% Triton X-100, 10 mM DTT, and 10% DMSO. The Triton X-100 and DTT were added to the assay buffer on the day of the experiment. The DMSO originated from the addition of NS4A peptide and inhibitor to the solution.

2.2. Expression and purification of NS3 proteases

2.2.1. Generation of NS3 protease expression plasmids

The coding sequence of the genotype 1b (con-1 strain) HCV NS3 protease domain was PCR amplified from a plasmid encoding the I389luc-ubi-neo/NS3-3'/ET replicon (Reblikon, Mainz, Germany) [4]. The 5'-PCR primer was designed to encode an N-terminal K_3 hexahistidine tag and to insert an in-frame rTEV protease cleavage site into the NS3 coding sequence. The resulting DNA fragment was cloned into the pET28 protein expression vector (Invitrogen, Grand Island, NY) yielding the p28-N6H-Tev-NS3(181)1b.

The coding sequence of the genotype 2 (JFH-1 strain) HCV NS3 protease domain was generated from seven overlapping synthetic oligonucleotides based on the JFH-1 sequence [5]. PCR amplification was used to assemble the overlapping oligos using a 5'-PCR primer encoding an N-terminal hexahistidine tag to insert an in-frame rTEV protease cleavage site into NS3 and a downstream 3'-PCR primer. The resulting DNA fragment was cloned into pET28 yielding the expression vector p28-N6H-Tev-NS3(181)2a.

The coding sequence for the genotype 3 HCV protease domain was amplified by RT-PCR using a Titan One Tube RT-PCR Kit (Roche, Indianapolis, IN) and RNA extracted from HCV-positive human serum (BBI Diagnostics, West Bridgewater, MA) using a QIAmp UltraSens Virus Kit (Qiagen, Valencia, CA). The 5'-PCR primer was designed to encode an N-terminal hexahistidine tag and to insert an in-frame rTEV protease cleavage site into the NS3 protease coding sequence. The resulting DNA fragment was cloned into pET28 yielding the expression vector p28-N6H-Tev-NS3(181)3.

2.2.2. NS3 protease protein expression

BL21AI bacteria (Invitrogen) were transformed with genotype 1b, 2a, or 3 NS3 expression vectors and used to inoculate a 20 L fermentation vessel (Sartorius BBI Systems Inc., Bethlehem, PA), containing 18 L of fresh 2YT medium supplemented with 50 μg/mL kanamycin. When cell densities reached an OD_{600} of 1, the temperature of the cultures was reduced from 37 °C to 28 °C, $ZnSO_4$ was added to a final concentration of 30 μM, and induction was immediately initiated by the addition of 14 mM L-arabinose and 1 mM IPTG (final concentrations). Cells were harvested by centrifugation 4 hours post-induction and were stored as frozen pellets at −80 °C prior to NS3 protein purification.

2.3. Purification of NS3 proteases

2.3.1. Purification of genotype 1b NS3 protease

Bacterial pellets were resuspended and lysed in a buffer containing 25 mM Tris pH 7.6, 300 mM NaCl, 0.1% CHAPS, and 10% glycerol using a microfluidizer. Homogenates were clarified by centrifugation at 48,000 × g for 1 hour, and the supernatant was loaded onto a 5 mL HisTrap-HP column (GE Healthcare) that had been equilibrated in lysis buffer with 50 mM imidazole. After the column was washed extensively to remove non-specifically bound contaminant proteins, the histidine-tagged NS3(1–181) protein was eluted from the column

using a linear imidazole gradient in lysis buffer. Fractions containing NS3(181) proteins (Ni pool) were pooled, and the Ni pool was diluted into SP A Buffer (25 mM Tris pH 7, 0.1% CHAPS, 10% glycerol, 2 mM DTT) to be fractionated on a SP-HiTrap column (GE Healthcare) using a linear salt gradient. Fractions eluted from the SP-HiTrap column at NaCl concentrations centered at 300 mM were pooled, frozen as small aliquots, and stored at −80 °C.

2.3.2. Purification of genotype 2a and genotype 3 NS3 proteases

Bacterial pellets collected from the expression of genotype 2a or 3 HCV NS3 proteases were homogenized in lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1 mM PMSF) and passed through a microfluidizer at 18,000 pounds/in^2. Homogenized cell lysates were centrifuged at 30,000 × *g* for 30 min at 4 °C. The resulting P1 pellets were washed with wash buffer I (25 mM Tris, pH 7.5, 1% CHAPS) followed by centrifugation at 10,000 × *g* for 30 min at 4 °C. The resulting P2 pellets were washed with wash buffer II (50 mM CAPS buffer, pH 10.8, 2 M NaCl, 2 M urea) followed by centrifugation at 30,000 × *g* for 30 min at 4 °C. The resulting P3 pellets were resuspended in 20 mL of solubilization buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 8 M urea) and incubated at 4 °C for 1 hour. Solubilized proteins were passed through a 0.45 micron filter. Protein concentrations were measured, and the solutions were adjusted to 40 mM DTT, incubated for 30 min at 4 °C and then quickly diluted into refolding buffer (25 mM Tris, pH 8.5, 0.8 M guanidine-HCl, 0.4 M L-arginine, 10 mM $ZnSO_4$) while stirring. Protein solutions were incubated at 4 °C overnight to allow refolding. Refolded proteases were centrifuged at 30,000 × *g* for 10 min to remove residual precipitates. Final protein concentrations were then measured, and the NS3 proteases were aliquoted, snap frozen in liquid nitrogen, and stored at −80 °C.

2.4. HCV NS3 protease IC_{50} determination

HCV NS3 protease activity was monitored using a fluorescence resonance energy transfer depsipeptide substrate (RET S1; Anaspec) based on the method of Taliani [6]. Briefly, 2–10 nM of purified NS3 protease domains were preincubated at 37 °C for 10 min with 20 μM isogenic NS4A peptide cofactors (Sigma, St. Louis, MO) in 40% glycerol buffer with 50 mM HEPES pH 7.5 and 10 mM DTT. Compounds were diluted serially 1:3 in DMSO and pre-incubated with the enzyme/cofactor mixture for 10 min. Reactions were started by the addition of 2 μM RET S1 substrate (final concentration). Fluorescence increase was measured continuously over 1 hour using a Victor^3 V fluorescence plate reader (Perkin Elmer, Waltham, MA). Initial velocities were calculated for each inhibitor concentration using Workout 1.5 software (DAZDAQ, East Sussex, UK) with the maximal slope algorithm. Velocity data were converted into percentages relative to the untreated control (defined as 100%), and nonlinear regression was performed to calculate IC_{50} values.

2.5. Measurement of K_i values

Compound dilutions were made in DMSO at 20× final concentration. Reaction mixtures were prepared in 96-well assay plates. A solution of enzyme and NS4A peptide in assay buffer (25 μL volume with both reagents at 4× final concentration) was mixed with 45 μL assay buffer and 5 μL of either inhibitor or DMSO, and pre-incubated for 1 hour. The reaction was started by addition of 25 μL substrate solution at 4× final concentration. After the addition of substrate, plates were mixed vigorously for 5–10 s on a plate shaker, and fluorescence was measured every 30 s for 3 hours using a Tecan Safire 2 multimode plate reader with an excitation wavelength of 340 nm and an emission wavelength of 490 nm. The final assay solutions contained 50 pM NS3 protease, 20 μM NS4A peptide, and 4 μM substrate. The final inhibitor concentrations varied from 100 nM to 5 pM (GS-9256, vedroprevir, ciluprevir, danoprevir) or 5 μM to 250 pM (telaprevir) in 3-fold dilutions. No-inhibitor controls were included. In addition, wells containing no enzyme were used to monitor uncatalyzed hydrolysis of substrate.

Rates were calculated from the progress curves at steady-state, which was achieved in the time frame of 60–90 min after addition of substrate. To determine the inhibitor potency (K_i or K_i^*), rates were plotted as a function of inhibitor concentration, and the data were fit with Eq. (1) [7], where v is the measured reaction rate in the presence of inhibitor, v_0 is the rate in the absence of inhibitor, $[E]_t$ and $[I]_t$ are the total concentrations of enzyme and inhibitor in solution, and K_i^{app} is defined in Eq. (2). For those compounds that showed two-step binding (see below), the potency derived from the steady-state inhibition experiments is denoted as K_i^*.

$$\frac{v}{v_0} = \frac{[E]_t - [I]_t - K_i^{app} + \sqrt{\left([E]_t - [I]_t - K_i^{app}\right)^2 + 4[E]_t K_i^{app}}}{2[E]_t} \quad (1)$$

$$K_i^{app} = K_i\left(1 + \frac{[S]}{K_m}\right) \quad (2)$$

2.6. Measurement of association kinetics

The onset of inhibition of NS3 protease was measured using an Applied Photophysics (Surrey, UK) SX20 stopped-flow spectrometer. The instrument was operated in fluorescence mode, with the excitation wavelength set to 340 nm and emission detected using a 395-nm high-pass optical filter. The temperature of the reaction chamber was set to 20 °C using a circulating water bath. To avoid a fluorescence change associated with the physical mixing of the NS3 protease and the fluorescent substrate, the experiments were performed using sequential mixing. Separate solutions of enzyme, substrate, and inhibitor were prepared in reaction buffer containing 20 μM NS4A peptide. To initiate the reaction, enzyme and substrate solutions in separate drive syringes were mixed in an aging tube. After a delay time of 30 s, the inhibitor was mixed with the solution of enzyme and substrate, and the fluorescence was monitored continuously for 500 s. The final reagent concentrations were 2 nM NS3 protease, 20 μM NS4A peptide, and 4 μM substrate. The final inhibitor concentration was varied from 1 μM to 7.8 nM using 2-fold dilutions.

The data analysis for the onset of inhibition followed established methodology [8,9]. Progress curves were fit using Eq. (3), where [P] is the concentration of product, v_0 is the initial rate prior to addition of inhibitor, v_s is the steady-state rate in the presence of inhibitor, and k is the observed rate of inhibitor binding. Plots of k versus [I] were generated and fit with models for one-step and two-step inhibitor binding. For the one-step model, the data were fit using Eq. (4), where the parameters are defined as in Fig. 2. For the two-step model, the data were fit with Eq. (5), where k_3, k_4, and K_i are defined as in Fig. 2, and [S] and K_m refer to the fluorescent substrate. For fits using either of the models, the y-intercept was poorly determined, and therefore the calculated values k_2 and k_4 from this analysis were considered unreliable. The values for k_2 and k_4 were determined from the inhibitor dissociation kinetics. The relationship between K_i and K_i^* for compounds with two-step binding is given in Eq. (6).

$$[P] = v_s t + \left(\frac{v_0 - v_s}{k}\right) \cdot \left(1 - e^{-kt}\right) \quad (3)$$

$$k = k_2 + \frac{k_1[I]}{1 + ([S]/K_m)} \quad (4)$$

$$k = k_4 + k_3\left[\frac{[I]/K_i}{1 + ([S]/K_m) + ([I]/K_i)}\right] \quad (5)$$

$$K_i^* = \frac{K_i k_4}{k_3 + k_4} \quad (6)$$

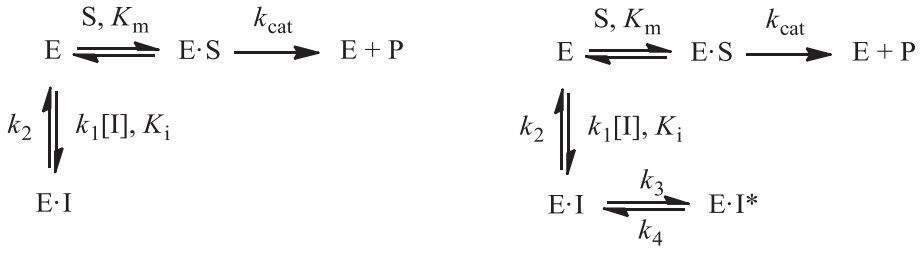

Fig. 2. One-step (left) and two-step (right) binding mechanisms.

2.7. Measurement of dissociation kinetics

Solutions of equimolar concentrations of NS3 protease and inhibitor (10 nM for GS-9256, vedroprevir, and ciluprevir; 5 nM for danoprevir) in reaction buffer supplemented with 20 μM NS4A peptide in a total volume of 100 μL were incubated at room temperature for 1 hour. Aliquots (10 μL) were then diluted 100-fold in reaction buffer containing 20 μM NS4A peptide. At time points over the time range of 0–30 hour, 50 μL aliquots of the diluted enzyme-inhibitor solution were removed and mixed with 50 μL of a solution of fluorescent substrate and 20 μM NS4A peptide in reaction buffer. Final concentrations were 25 or 50 pM NS3 protease, 20 μM NS4A, 25 or 50 pM inhibitor, and 4 μM substrate. Reactions were monitored for 15 min using a Tecan Safire 2 multimode plate reader in fluorescence mode with an excitation wavelength of 340 nm and an emission wavelength of 490 nm. Rates were measured as the slope of the progress curve and were corrected for background substrate hydrolysis. Reactions were performed in triplicate.

Dissociation rate data were fit using the model shown in Eq. (7). The derived rate constant applies equally well to one- and two-step binding models. An analytic rate expression for this dissociation, taking into account the change in the concentration of free inhibitor over the course of the reaction, has been derived previously [10]. The solution can be written as in Eq. (8), where [E] is the concentration of free enzyme, $[E]_\infty$ is the concentration of free enzyme at equilibrium, $[E]_{total}$ is the total concentration of enzyme in solution, α is defined in Eq. (9), and k is equal to k_2 or k_4, depending on the mechanism of inhibition. For the tight-binding compounds GS-9256, vedroprevir, ciluprevir, and danoprevir, this equation was used to fit the data. In this data analysis, it was assumed that the addition of substrate did not perturb the binding equilibrium of enzyme and inhibitor significantly over the time frame of the enzyme activity measurement. This assumption is consistent with the slow dissociation kinetics of the inhibitors.

The dissociation kinetics for telaprevir were measured as above, except that the initial pre-incubation used 10 nM NS3 protease and 100 nM telaprevir. Because $[E]_{total} << [I]_{total}$ in this experiment, the data were fit using Eq. (10), the first derivative form of Eq. (3), where k is defined in Eq. (4) and (5) [8].

$$E \cdot I \underset{k'}{\overset{k}{\rightleftharpoons}} E + I \tag{7}$$

$$[E] = \frac{[E]_\infty \left(1 - e^{-\alpha k t}\right)}{1 + \left(\frac{[E]_{total} - [E]_\infty}{[E]_{total}}\right) e^{-\alpha k t}} \tag{8}$$

$$\alpha = \frac{\left(2[E]_{total} - [E]_\infty\right)}{[E]_\infty} \tag{9}$$

$$v = v_s + (v_0 - v_s) e^{-kt} \tag{10}$$

2.8. Selectivity for mammalian proteases

2.8.1. Human leukocyte elastase (HLE) inhibition assay

Compounds including a positive control (Elastase Inhibitor III; Calbiochem, San Diego, CA) were diluted serially 1:3 in DMSO in polypropylene 96-well plates. Ten microliters of compounds were transferred to black, clear bottom 96-well assay plates (Costar, Lowell, MA). HLE enzyme (6 nM; Calbiochem) was added to assay plates in a buffer containing 50 mM Tris pH 7.4 and 100 mM NaCl and incubated for 10 min at 37 °C. Reactions were initiated by adding Elastase V substrate (Calbiochem) prepared in 50 mM Tris buffer to a final concentration of 350 μM in a final reaction volume of 100 μL. Reactions were monitored continuously over 1 hour at 37 °C using an excitation wavelength of 380 nm and an emission wavelength of 460 nm in a Gemini SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). Initial velocities were calculated using the instrument's SoftMax software, and linear regression was performed to calculate IC_{50} values.

2.8.2. Porcine pancreatic elastase (PPE) inhibition assay

Compounds including a positive control (Elastase Inhibitor III) were serially diluted in DMSO and transferred to black, clear bottom 96-well assay plates as described in Section 2.8.1. Elastase substrate V was added to the assay plates at a final concentration of 1 mM in 50 mM Tris buffer, pH 8 in an 80 μL volume and incubated for 10 min at 37 °C. PPE enzyme (5 nM; Calbiochem) was added to the plates to initiate the reaction and bring the final volume to 100 μL. Reactions were monitored, and IC_{50} values were calculated as described in Section 2.8.1.

2.8.3. Proteinase 3 (Pr3) inhibition assay

Compounds including a positive control (Elastase Inhibitor III) were serially diluted in DMSO and transferred to black, clear bottom 96-well assay plates as described in Section 2.8.1. Pr3 enzyme (100 nM; Athens Research and Technology, Athens, GA) was added to assay plates in a buffer containing 50 mM Tris pH 7.4 and 100 mM NaCl and was incubated for 10 min at 37 °C. Reactions were initiated by adding Elastase V substrate prepared in 50 mM Tris buffer to a final concentration of 250 μM in a final reaction volume of 100 μL. Reactions were monitored, and IC_{50} values were calculated as described in Section 2.8.1.

Table 1
Inhibition of genotype 1 NS3/4A protease by of GS-9256, vedroprevir, and comparators.

Compound	K_i (nM)[a]		
	Genotype 1b[b]	Genotype 2a[c]	Genotype 3[c]
GS-9256	0.089 ± 0.012	3.8 ± 1.4	104 ± 10
Vedroprevir	0.41 ± 0.12	39 ± 7	319 ± 24
Ciluprevir	0.038 ± 0.022	3.3 ± 2.0	13 ± 7
Telaprevir	7.9 ± 3.1	17 ± 1.4	76 ± 25

Values represent the mean ± SD of 3 or more independent experiments.
[a] All steady-state potency values are reported as K_i values in this table.
[b] 50 pM solution of NS3 protease was pre-incubated with compounds for 1 hour before addition of substrate during the K_i assay.
[c] 2 nM solution of NS3 protease was pre-incubated with compounds for 10 min before addition of substrate during the IC_{50} assay.

Table 2
Kinetic parameters of HCV NS3 protease inhibitors.

	K_i (M)	k_1 (M^{-1} s^{-1})	k_2 (s^{-1})
GS-9256	$(8.9 \pm 1.2) \times 10^{-11}$	$(4.0 \pm 2.1) \times 10^{5}$	$(4.8 \pm 0.5) \times 10^{-5}$
Vedroprevir	$(4.1 \pm 1.2) \times 10^{-10}$	$(1.00 \pm 0.03) \times 10^{6}$	$(2.6 \pm 1.3) \times 10^{-4}$

	K_i^* (M)	K_i (M)	k_3 (s^{-1})	k_3/K_i (M^{-1} s^{-1})	k_4 (s^{-1})
Ciluprevir	$(3.8 \pm 2.2) \times 10^{-11}$	$(3.0 \pm 2.7) \times 10^{-7}$	0.28 ± 0.08	$(1.32 \pm 0.90) \times 10^{6}$	$(1.4 \pm 0.2) \times 10^{-4}$
Danoprevir	$<1 \times 10^{-11}$	$(1.5 \pm 0.3) \times 10^{-7}$	0.33 ± 0.04	$(2.3 \pm 0.2) \times 10^{6}$	$<1 \times 10^{-5}$
Telaprevir	$(7.9 \pm 3.1) \times 10^{-9}$	$(1.21 \pm 0.08) \times 10^{-6}$	$(2.4 \pm 0.2) \times 10^{-3}$	$(1.9 \pm 0.1) \times 10^{3}$	$(3.4 \pm 0.7) \times 10^{-5}$

Compounds are grouped into those with one-step binding (top) and two-step binding (bottom).
Values represent the mean ± SD of 2–5 independent experiments.

2.8.4. Cathepsin L (CatL) inhibition assay

Compounds including a positive control (Cathepsin L inhibitor II; Calbiochem) were serially diluted in DMSO and transferred to black, clear bottom 96-well assay plates as described in Section 2.8.1. CatL enzyme (7.5 nM; Sigma) was added to assay plates in a buffer containing 300 mM sodium acetate pH 5.5, 4 mM EDTA, and 8 mM DTT and was incubated for 10 min at 37 °C. Reactions were initiated by adding Z-Phe-Arg-AMC * HCl substrate (Bachem, Torrance, CA) prepared in DMSO to a final concentration of 2 μM in a final reaction volume of 100 μL. Reactions were monitored, and IC_{50} values were calculated as described in Section 2.8.1.

2.8.5. Cathepsin D (CatD) inhibition assay

Compounds including a positive control (Pepstatin A; Calbiochem) were serially diluted in DMSO and transferred to black, clear bottom 96-well assay plates as described in Section 2.8.1. CatD enzyme (4.8 nM; Calbiochem) was added to assay plates in a 50 mM sodium acetate buffer, pH 4, and was incubated for 10 min at 37 °C. Reactions were initiated by adding Cathepsin D/E Substrate (Calbiochem) prepared in 50 mM sodium acetate buffer to a final concentration of 6 μM in a final reaction volume of 100 μL. Reactions were monitored as described in Section 2.8.1 but using an excitation wavelength of 320 nm and an emission wavelength of 393 nm. Reactions were monitored, and IC_{50} values were calculated as described in Section 2.8.1.

3. Results

3.1. Determination of inhibitor potency

GS-9256, vedroprevir, and comparator compounds were tested for their ability to inhibit genotype 1b, 2a, and 3 wild-type NS3 proteases in enzymatic assays performed using recombinant NS3 protease domains and isogenic NS4A peptide cofactors provided in *trans*. IC_{50} values were measured and converted to K_i values using the Cheng-Prusoff equation (Eq. (2), where IC_{50} is equivalent to K_i^{app}). For genotypes 2a and 3, this analysis was sufficient to obtain accurate K_i values. For the initial experiments using genotype 1b protease, the IC_{50} value was approximately the same as the enzyme concentration, and the classical determination of K_i was deemed insufficient. The binding affinity of vedroprevir, GS-9256, and comparator compounds for genotype 1b protease was determined through the use of a lower enzyme concentration and equations for tight-binding inhibition.

Measured K_i values against the three genotypes are shown in Table 1. GS-9256 and vedroprevir were the most potent against the genotype 1b protease, with K_i values of 89 pM and 410 pM, respectively. GS-9256 K_i values were elevated for genotype 2a (43-fold) and more markedly elevated for genotype 3 (1200-fold). Vedroprevir K_i values were markedly elevated for genotype 2a and 3 NS3/4A proteases (95- and 780-fold, respectively) compared to genotype 1b NS3/4 protease

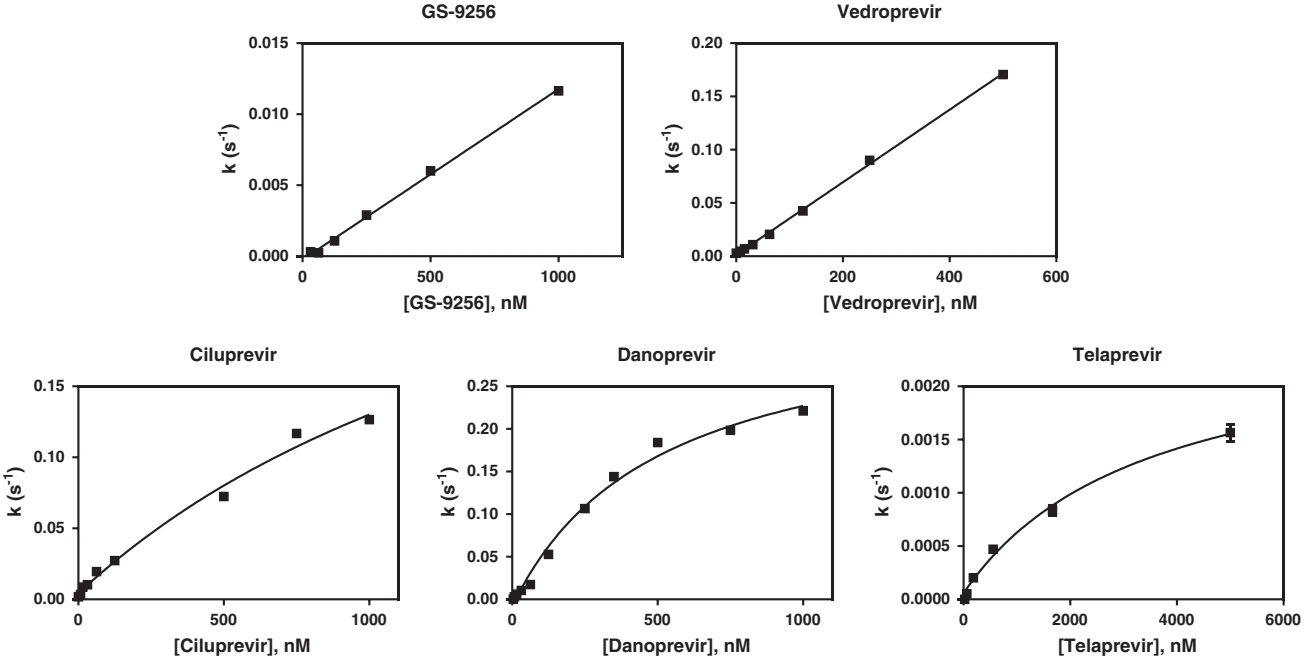

Fig. 3. *k* versus inhibitor concentration for the binding of HCV protease inhibitors.

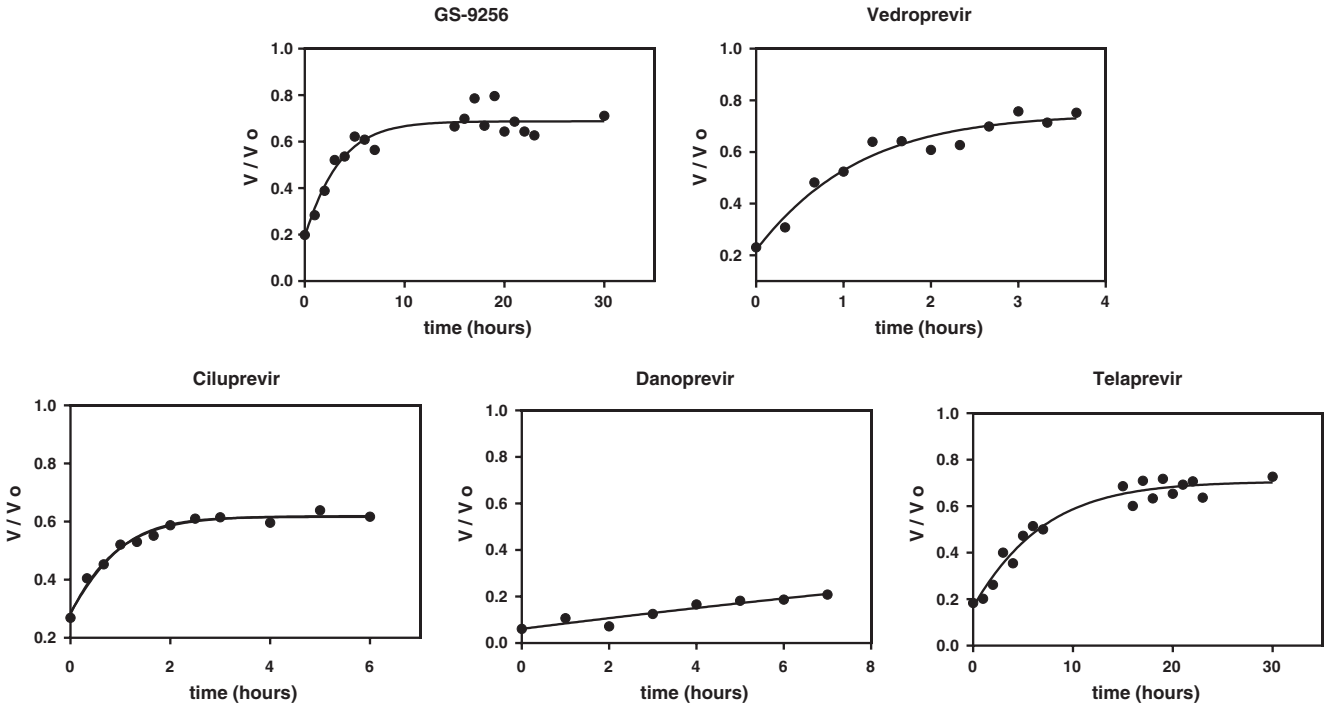

Fig. 4. Dilution recovery curves. Rates are normalized to uninhibited enzyme.

K_i values. Similarly, the control compounds ciluprevir and telaprevir each showed greatest potency against the genotype 1b enzyme, with weaker inhibition of the genotype 2a and 3 proteases.

Of the compounds tested against the genotype 1b enzyme, the macrocyclic inhibitors GS-9256, danoprevir, and ciluprevir were the most potent compounds in this study, all with K_i values <100 pM (Tables 1 and 2). The acyclic inhibitors showed weaker inhibition: vedroprevir had a K_i value of 410 pM, and telaprevir had a K_i value of 7900 pM. The K_i value measured for ciluprevir (38 pM) was lower than the value of 660 pM originally reported for this compound [11], whereas the K_i values for danoprevir and telaprevir were in agreement with values published previously [12,13]. Because steady-state inhibition experiments cannot differentiate between one- and two-step binding mechanisms, all potency values in Table 1 are presented as K_i values for simplicity.

3.2. Association and dissociation kinetics

The kinetics of compound binding and dissociation from the 1b enzyme were measured for GS-9256, vedroprevir, and the three comparator compounds. Association kinetics were measured using a stopped-flow apparatus to ensure that the kinetics of mixing did not confound data analysis. Plots of k versus [I] allowed a comparison of one-step and two-step binding mechanisms and determination of associated rate constants. Linear plots suggest simple second-order kinetics and a one-step binding mechanism. Hyperbolic plots imply a two-step binding process. When hyperbolic plots were observed, the K_i value (two-step model) was determined using Eq. (5). For the HCV protease inhibitors in this study, plots of k versus [I] are given in Fig. 3, and association rate constants are shown in Table 2. The association rate constants k_1 or k_3/K_i were generally similar across compounds. Among GS-9256, vedroprevir, ciluprevir, and danoprevir, the highest and lowest rate constants differed by a factor of only 5.8. Telaprevir, a covalent, reversible inhibitor, showed markedly slower association kinetics because of both weak pre-binding and slow formation of the covalent adduct. The association rate constants reported in this study for ciluprevir, danoprevir, and telaprevir are higher than those reported in previous literature [12,14].

Progress curves for the recovery of activity are shown in Fig. 4, and dissociation rate constants are given in Table 2. Dissociation rate constants for the tested compounds spanned a range of $<1 \times 10^{-5}$ s^{-1} to 3×10^{-4} s^{-1}. Danoprevir, the compound with the lowest k_4 value, was also the most potent inhibitor. However, as highlighted by telaprevir, both the association and dissociation kinetics were important in differentiating the binding affinities of the compounds. The binding affinities measured from steady-state kinetics were in good agreement with the association and dissociation rate constants.

3.3. Selectivity for mammalian proteases

To investigate relative selectivity for NS3 protease versus host proteases, GS-9256, vedroprevir, and comparator compounds were tested for their ability to inhibit five mammalian proteases including three serine proteases (HLE, PPE, and Pr3), the aspartic protease (CatD), and the cysteine protease (CatL). Selectivity is summarized in Table 3 and is expressed as the fold difference between the average K_i for the mammalian protease and the average K_i for genotype 1b NS3 protease run in parallel experiments. GS-9256 showed >10,000- to almost a million-fold selectivity against all mammalian proteases tested. Vedroprevir showed >50,000- to almost 400,000-fold selectivity.

Table 3
Selectivity of GS-9256, vedroprevir, and comparators against mammalian proteases.

Compound	Selectivity–Fold of 1b NS3 Protease K_i				
	PPE (serine)	HLE (serine)	Pr3 (serine)	CatD (aspartic)	CatL (cysteine)
GS-9256	929,439	10,653	205,438	76,172	136,730
Vedroprevir	512,566	50,503	321,116	90,820	396,825
Ciluprevir	5,665,205	166,338	5,444,646	981,273	4,385,965
Telaprevir	7	88	43	3132	1491

CatD, cathepsin D; CatL, cathepsin L; HLE, human leukocyte elastase; PPE, porcine pancreatic elastase; Pr3, human proteinase.
Values represent the mean of 3 or more independent experiments.

4. Discussion

GS-9256 and vedroprevir are potent inhibitors of NS3 protease. Because of this potency and the potential for these compounds to have slow binding kinetics, we assessed inhibitor binding to genotype 1b NS3 protease through the use of steady-state inhibition as well as by the measurement of association and dissociation kinetics. Across the set of tested compounds, the K_i values determined from steady-state experiments were consistent with the potency values predicted from kinetic data. For both GS-9256 and vedroprevir, potency was greatest against genotype 1b enzyme, followed by genotype 2a and 3 enzymes. This is in accord with the trends observed in cellular HCV replicon assays. For GS-9256, EC_{50} values of 20, 280, and >32,000 nM were measured using genotype 1b, 2a, and 3 replicons, respectively ([15] and unpublished data). Similarly, EC_{50} values of 1.8, 320, and 2000 nM were measured for vedroprevir against the 1b, 2a, and 3 genotypes, respectively [2,16].

Binding kinetics were determined both as a confirmation of potency values and to provide an understanding of the binding mechanism. Because potent inhibitors may also have slow binding kinetics, it is possible that steady state may not be achieved during the preincubation and reaction times used in standard enzymatic assays. By comparing the association and dissociation rate constants with the measured K_i or K_i^* values, one can establish whether the data sets are consistent. Specifically, the ratio of the dissociation and association rates (k_2/k_1 for one-step binding; $K_i k_4/(k_3 + k_4)$ for two-step binding) should agree with the K_i values determined from steady-state experiments. For all of the compounds tested, the two measures of potency agreed to within a factor of three, demonstrating consistency of the data.

The association kinetics of both GS-9256 and vedroprevir showed a linear relationship between k and [I], suggesting that a one-step mechanism best describes the binding of these compounds. One-step binding has also been reported for the NS3 inhibitor faldprevir (BI-201335) [17], an acyclic compound with a carboxylic acid as a non-covalent, charged warhead. Ciluprevir has also been reported to bind with a one-step mechanism [14], whereas the data presented in this work suggest the compound could have a one- or two-step mechanism. In contrast, compounds containing ketoacid or ketoamide warheads show kinetics consistent with two-step binding [14,18]. Because these classes of compounds are covalent modifiers of the active-site serine residue in NS3, the two-step binding is assumed to consist of pre-binding to the active sites, followed by slower covalent modification of the serine. As reported elsewhere [12] and confirmed in this study, the acylsulfonamide-containing inhibitor danoprevir also displays two-step binding, although the molecular basis of the two-step binding has not been established.

Although NS3 protease does not have high sequence similarity to any human proteins, GS-9256 and vedroprevir were tested against a panel of mammalian proteases to assess potential off-target liabilities. For both compounds, selectivity against all proteases was >10,000-fold, suggesting that inhibition of host proteases is not likely to be a significant off-target liability in patients.

In summary, GS-9256 and vedroprevir are potent inhibitors of HCV protease, particularly the genotype 1b enzyme. They have rapid association kinetics and slow dissociation rates, and have little potential to inhibit host proteases. The value of NS3 protease inhibitors in combination with interferon and ribavirin for the treatment of hepatitis C has been demonstrated with boceprevir, telaprevir, and simeprevir. GS-9256 and vedroprevir have the potential for use in more advanced, interferon-sparing therapies. Because of the likely development of viral resistance when used as monotherapy, optimal therapies utilizing GS-9256 or vedroprevir would also include compounds targeting other viral proteins. For instance, a combination of vedroprevir with the NS5A inhibitor ledipasvir and the NS5B inhibitor sofosbuvir was recently shown to give a very rapid drop in viral load and a 100% sustained viral response rate after only six weeks of therapy in a small pilot study (n = 10 patients) [19]. The properties of GS-9256 and vedroprevir highlight the utility of these compounds as anti-HCV agents.

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