Investigating the Origin of the Slow-Binding Inhibition of HCV NS3 Serine Protease by a Novel Substrate Based Inhibitor

Yaya Liu,* Ayda Saldivar, Joshua Bess, Larry Solomon, Chih-Ming Chen, Rakesh Tripathi, Leo Barrett, Paul L. Richardson, Akhteruzzaman Molla, William Kohlbrenner, and Warren Kati

Antiviral Department, Infectious Disease Research and Advanced Technology, Pharmaceutical Discovery, Abbott Laboratories, Abbott Park, Illinois 60064-6217

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ABSTRACT: Indandiones were identified as a novel class of small molecule inhibitors of hepatitis C virus NS3 serine protease from high throughput screening. We further studied the structure activity relationships and the mechanisms of inhibition for this class of compounds. Our studies revealed two similar, yet different, mechanisms accounting for the apparent indandione inhibition of HCV NS3 protease. In one case, the apparent inhibition results from the chemical breakdown of the parent compound and the subsequent redox chemistry of the compound. Oxidation of the cysteine containing substrate A to a disulfide-linked dimer converts this substrate to a potent, slow-binding inhibitor with a K_i value of 170 nM. The second class of indandiones appears to react directly with the substrate to form an *S*-phenyl disulfide adduct with the P1 cysteine. This modification converts the substrate to a slow-binding inhibitor with a K_i value of 110 nM, a $k_{on} = 2370\ M^{-1}\ s^{-1}$, and $k_{off} = 2.5 \times 10^{-4}\ s^{-1}$. A stable analogue of this latter compound was synthesized that contained a CH_2-S linkage instead of the S−S linkage. The CH_2-S compound showed no inhibition at concentrations as high as 40 μM, which suggests an important role for the S−S linkage in the inhibitory mechanism. Cysteine 159, which lies near the active site of the HCV protease, was mutated to serine. The C159S mutant displayed wild-type catalytic activity and susceptibility to inhibition by the S−S linked inhibitor. This result argues against a mechanism involving disulfide exchange between the inhibitor and the sulfhydryl group of C159. The mechanism of inhibition for this S−S linked substrate based inhibitor is likely due to oxidation of cysteines involved in chelation of the structural zinc atom.

Hepatitis C virus is the major etiological agent of non-A, non-B hepatitis. The disease is a major health problem with an estimated 170 million people infected worldwide. HCV[1] infection results in mild and acute liver disease, but chronic infections are common and may eventually develop into liver cirrhosis or hepatocellular carcinoma. HCV is the second most common etiological agent in the development of hepatocellular carcinoma (*1*−*5*). The treatments currently available for chronic HCV infection are interferon (IFN)-α, pegylated interferon, or one of these agents in combination with ribavirin. However, these treatments show low clinical efficacy and insufficient suppression of HCV replication, demonstrating an urgent need for the development of more effective antiviral drugs (*6*, *7*).

The predominant focus of drug discovery efforts in recent years has been directed toward the serine protease activity of the HCV NS3 protein, which is required for the post-translational processing of the nonstructural region of the HCV polyprotein. The N-terminal one-third domain of the NS3 gene encodes a serine protease that performs all cleavages downstream of NS3. This protease domain also utilizes NS4A as a cofactor for the 4A/4B, 4B/5A, and 5A/5B cleavages (*8*−*10*). Several groups have independently solved the crystal structure of the recombinant protease (*11*, *12*) that confirms its chymotrypsin-like serine protease mechanism with a Ser−His−Asp active site triad. The crystal structure also reveals the following structure features about the HCV NS3 serine protease. The active site of the HCV protease sits on the surface of the protein and is quite featureless and solvent exposed. NS4A, a 54-amino acid peptide, is an essential cofactor for NS3, and it forms a detergent-stable noncovalent complex with NS3. A tetrahedrally coordinated zinc ion complexed with three Cys-residues and with a His-residue is found in the C-terminal domain of NS3 serine protease. Given the distance of the zinc ion from the catalytic serine residue (>20 Å), it likely plays a structural and not a catalytic role. Therefore, NS3 protease inhibitor discovery has diverged into three major efforts: (1) targeting the traditional active site Ser−His−Asp triad, (2) targeting disruption of the NS4a cofactor binding, and (3) targeting the structural zinc binding site (*13*). The later two approaches are technically challenging because of the high affinity and extensive interaction of the NS3/NS4A complex and the rigorous specificity requirement for zinc ion chelating to avoid cellular toxicity. Substrate-based peptide inhibitors targeting the active site have been reported as competitive inhibitors (*14*−*18*). These substrate-based

* Corresponding author. Telephone: (847) 937 8626. Fax: (847) 938 2756. E-mail: yaya.liu@abbott.com.

[1] Abbreviations: HCV, hepatitis C virus; HCMV, human cytomegalovirus; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; MALDI/MS, matrix assisted laser desorption ionization mass spectroscopy.

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inhibitors include noncleavable substrates, N-terminal cleavage products, and transition state analogues. Some protein and nucleic acid based macromolecules have also been reported (*19*−*23*). However, because they are peptide based or nucleic acid based molecules with a relatively high molecular weight, the effective delivery and poor bioavailability become disadvantageous. Several classes of small molecule NS3 protease inhibitors have been reported that include phenanthrenequinone (*24*), thiazolidines (*25*), benzamides (*26*), and benzoylamides (*27*). Most of these compounds have been shown to be noncompetitive inhibitors with poor NS3 protease selectivity over other serine proteases.

During our screening for small molecule inhibitors of HCV NS3 protease, we identified several indandiones as submicromolar inhibitors from our chemical library. The novel indandione core structure led us to further characterize the mechanism of inhibition of this class of compounds. To our surprise, we found that two different mechanisms were responsible for the apparent indandione mediated inhibition of HCV NS3 serine protease. In one class, the apparent inhibition is due to the redox chemistry of the compound and subsequent formation of the dimeric disulfide substrate involving the P1 cysteine. In the other case, the inhibition is due to a newly formed substrate based inhibitor. In both cases, the substrate becomes modified with a disulfide-linked adduct on the P1 cysteine to yield potent slow-binding inhibitors of HCV NS3 protease. The origin of slow binding was investigated in this work. The mechanism of inhibition for these substrate based inhibitors is significantly different from previously reported substrate based peptide inhibitors.

MATERIALS AND METHODS

Substrates. The peptide substrate, Ac-GE(EDANS)NHCH$_2$-CH$_2$OCH$_2$CH$_2$O-CH$_2$CH$_2$COEDVVACSMSYNHCH$_2$CH$_2$-OCH$_2$CH$_2$OCH$_2$CH$_2$CO-K(DABCYL)G-NH$_2$ (A), which mimics the P6−P4′ region of NS5A-5B junction and uses the EDANS and DABCYL pair as the fluorescence resonance energy transfer system, was synthesized on an automated multiple peptide synthesizer (Applied Biosystems Inc., model 430A). The glycol linker was introduced to increase the solubility of this peptide. The peptide was purified by PrepPAK C-18 preparative reverse-phase HPLC (25 × 300 mm, 15 micron, 100 Å, Waters) to a purity greater than 95% as judged by analytical HPLC. The HCV NS3 protease fluorogenic depsipeptide substrate, Ac-DED(EDANS)EEAbu-ψ[COO]ASK(DABCYL)-NH$_2$ (B), whose structure is based on the cleavage site of 4A/4B, was obtained from AnaSpec (San Jose, CA). The purity of this peptide was greater than 95% as judged by analytical HPLC.

Inhibitors. The dimeric disulfide substrate was obtained by air oxidation of the substrate in 50 mM triethanolamine buffer (pH = 7.4) at room temperature overnight. The dimeric disulfide substrate eluted at 7.1 min, and substrate A eluted at 7.8 min in a 30−50% acetonitrile gradient in H$_2$O/0.1% TFA at a flow rate of 1 mL/min for 13 min on Lichrospher C-18 reverse-phase column (4 × 125 mm, 5 micron, Merck). The inhibitor 1 (see Scheme 4) was synthesized by mixing a 10-fold molar excess of compound **7** with 50 μM 5A/5B amide substrate A in 50 mM triethanolamine buffer (pH = 7.4) at room temperature. The resulting *S*-phenyl substrate adduct was purified by preparative HPLC using a Lichrospher C-18 reverse-phase column (4 × 125 mm, 5 micron, Merck) and a 15−45% acetonitrile gradient in H$_2$O/0.1% TFA at a flow rate of 1 mL/min for 23 min. The substrate eluted at 18 min, and the inhibitor 1 eluted at 21 min. The identities of the substrate and *S*-phenyl substrate adduct were confirmed by MALDI/MS mass spectroscopy with M + 2Na$^+$ of 2349 for substrate A and 2458 for the inhibitor 1.

Enzymes. The first 181 amino acids of the NS3 polypeptide (ΔNS3) region of the HCV genome (BK strain, 28) were selected for expression of the active enzyme in *Escherichia coli*. The NS4A peptide with the sequence of KKGSVVIVLSGKPAIIPKK was purchased from Peptidogenic (Livermore, CA). Another HCV NS3 protease construct, with NS4A fused cis to the C-terminus of NS3, was also used in this study. This NS3−NS4A fusion protein includes glutathione-*S*-transferase at the N-terminus and is fused to the first 204 amino acids of the NS3 polypeptide, which, in turn, is fused to the complete 54 amino acids of the NS4A protein (GST-ΔNS3-NS4A) (*29*). The GST-ΔNS3-NS4A was purified using glutathione sepharose chromatography. The GST-ΔNS3-NS4A protease is about 60% pure with GST as the major contaminating protein. The enzyme, which generated one set of cleavage products, confirms that only one protease was present in the preparations.

Construction of Wild-Type ΔNS3 and C159S ΔNS3. The construct used for expression of the ΔNS3 protease (BK strain) used in these studies was made from synthetic phosphorylated oligonucleotides and optimized for *E. coli* expression based on codon bias (*30*). The C159S ΔNS3 mutant was constructed using the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's recommendations using p28His-BK as a template and the primers C159S-S (5′-CTTCCGTGCTGCAGTTTCCACCCGGGGTGTTGCTAAAGCTGT) and C159S-AS (5′-ACAGCTTTAGCAAGACCCCGGGTGGAAACTGCAGCACGGAAG). The mutagenesis reaction also introduced a silent SmaI restriction endonuclease cleavage site for rapid screening. Plasmid DNA derived from transformants was isolated and sequence confirmed.

Expression of Wild-Type ΔNS3 and C159S Mutant ΔNS3. Plasmid DNA encoding the wild-type or mutant NS3 was used to transform competent BL21 (DE3) (Novagen, Madison, WI) to kanamycin resistance. The transformants were grown at 30 °C in Terrific Broth supplemented with 50 ug/mL kanamycin to an OD$_{600}$ of ∼1.0 and induced for 2 h at 30 °C with 1 mM IPTG. Cells were harvested by centrifugation.

Purification of Wild-Type ΔNS3 and C159S Mutant ΔNS3. Cell pellets were suspended on ice at 6 mL/gm cell paste using lysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 25% glycerol, 0.1% Triton-X100) supplemented with 0.5 mg/mL lysozyme, 0.05% β-mercaptoethanol, 5 mM imidazole, 1 mM PMSF, 5 ug/mL aprotonin, 1 ug/mL leupeptin, and 1 U/mL benzonase (EM industries). The suspension was passed through a French press and clarified by centrifugation at 20 000*g* for 30 min. The clarified supernatant was loaded onto a lysis buffer preequilibrated Ni-NTA metal affinity column (1 mL resin/gm of cell paste, Pro-Bind, Invitrogen) at a flow rate of 5 mL/min. The column was washed with 20 column volumes of buffer N (100 mM KPO$_4$, pH 7.0, 300 mM NaCl, 25% glycerol, and 7 mM 2-mercaptoethanol)

containing 25 mM imidazole. The ΔNS3 protease was batch eluted with 10 column volumes of the same buffer containing 300 mM imidazole. The Ni-NTA eluent was adjusted to 1.5 M $(NH_4)_2SO_4$ and allowed to stand at room temperature for 30 min. The solution was clarified by centrifugation at 20 000*g* for 30 min, and then the supernatant was applied to a Bio-Rad Macro-prep methyl HIC column (0.5 mL resin/gm of cell paste) at a flow rate of 5 mL/min. The column was washed with 10 volumes of 1.5 M $(NH_4)_2SO_4$ in buffer S (10 mM KPO_4, 10 mM dithiothreitol, and 25% glycerol). The ΔNS3 was eluted with 10 column volumes of 1.0 M $(NH_4)_2SO_4$ in buffer S. The eluted material was dialyzed exhaustively against buffer S and applied to an S-Sepharose ion exchange column (0.5 mL/gm of cell paste, Sigma) equilibrated in the same buffer. The column was washed with 10 column volumes of buffer S with 200 mM NaCl and eluted with a 10-column volume linear gradient of 200−700 mM NaCl in buffer S. Fractions containing NS3 were pooled and stored in 30% glycerol at −80 °C and used without further purification. Both wild-type and C159S mutant proteases are greater than 90% pure as judged by a Coomassie stained gel.

Plate Reader Fluorogenic Assay of HCV NS3 Protease Activity. Continuous assays were performed on an ICN Titertek Fluoroskan II 96-well microtiter fluorescence plate reader at ambient temperature using white microfluor U-bottom plates from Dynex, Inc. Excitation and emission wavelength filters of 355 and 485 nm, respectively, were used. Typically, 150 μL of assay buffer (50 mM triethanolamine, 20% glycerol, 50 mM NaCl, 3 mM $MgCl_2$, and 2.5 mM DTT, at pH = 7.4) with 10 μL of DMSO or inhibitor solution in DMSO was mixed with 20 μL of 25−250 μM substrate. DTT was omitted when the dimeric disulfide substrate and inhibitor 1 were tested for inhibition because of the thiol sensitive bonds contained within these compounds. Reactions were then initiated by the addition of 20 μL of HCV NS3 protease with a final enzyme concentration around 1 nM in the assay. In some cases, the inhibitor and enzyme were incubated for 45 min before the addition of the substrate. The time-dependent fluorescence intensity increase was monitored every 40 s for 30 min. Initial reaction velocities, expressed as fluorescence units per minute, were obtained by least-squares analysis of the initial phase of the reaction using Deltasoft data collecting and analyzing software (BioMetallics, Inc.).

HPLC Assay of Inhibition. A 144 μL aliquot of the reaction mixture from the plate assay was stopped by the addition of 3 μL of 10% TFA. A 3 μL aliquot of 0.05 mM free EDANS was added to function as an internal reference. A 100 μL aliquot of this mixture was injected onto a reverse phase HPLC to separate the reference free EDANS, cleaved EDANS peptide, and uncleaved substrate. The separation was accomplished using a Lichrospher C-18 reverse-phase column (4 × 125 mm, 5 micron, Merck) and a 10−50% acetonitrile gradient in H_2O/0.1% TFA at a flow rate of 1 mL/min for 10 min. A fluorescence detector with excitation wavelength at 242 nm and emission wavelength at 485 nm and a UV detector at 512 nm were used to quantify the amount of each of these three components. Inhibition was established by comparing the HPLC peak area ratio of cleaved EDANS peptide and free EDANS reference versus that for the uninhibited control.

Selectivity Assays. Fluorogenic peptides were also used as specific substrates for the quantitative measurement of other proteases as described previously (*31*, *32*). Assays were performed on an ICN Titertek Fluoroskan II 96-well microtiter fluorescence plate reader at ambient temperature using white microfluor U-bottom plates from Dynex, Inc. Excitation and emission wavelength filters of 370 and 460 nm, respectively, were used. Typically, 150 μL of assay buffer (50 mM Tris, 100 mM NaCl, 0.1% of Triton, pH = 7.5 for elastase; 50 mM Tris, 10 mM $CaCl_2$, 0.1% Triton, pH = 8.0 for chymotrypsin) was mixed with 10 μL of DMSO or inhibitor solution in DMSO and 20 μL of substrate so that the final substrate concentration was 25 μM. Reactions were then initiated by the addition of 20 μL of protease in assay buffer. Reactions were monitored for 30 min. Initial reaction velocities, expressed as fluorescence units per minute, were obtained by least-squares analysis of the initial phase of the reaction using Deltasoft data collecting and analyzing software (BioMetallics, Inc.).

IC_{50} and K_i Calculation. The percent inhibition was calculated from the initial rates of the inhibited reactions relative to the uninhibited control. IC_{50} values were calculated by fitting percent inhibition at six to eight inhibitor concentrations to the following eq 1. The K_i value was calculated from eq 2 using a predetermined K_m value for each specific substrate.

$$\% \text{ inhibition} = 100[I]/([I] + [IC_{50}]) \quad (1)$$

$$K_i = IC_{50}/(1 + [S]/K_m) \quad (2)$$

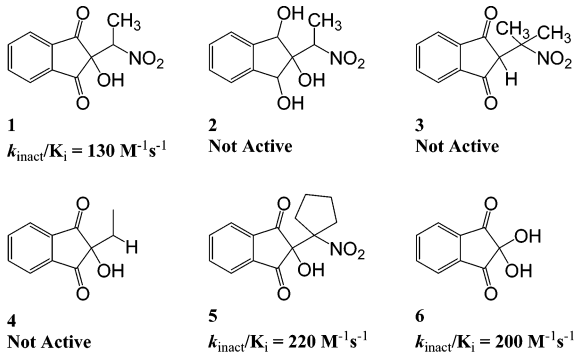
Slow-Binding Kinetics of S-Phenyl Substrate Adduct. Progress curves for slow-binding inhibitors are described by eq 3, where F_t is the fluorescence signal at various reaction times, V_0 is the reaction velocity at time zero, V_s is the final steady-state velocity, k_{obs} is the observed first-order rate constant for the approach to the steady state, and F_0 is the fluorescence signal at time zero (*33*, *34*).

$$F_t = F_0 + V_s t + (V_0 - V_s)(1 - \exp(-k_{obs} t)/k_{obs}) \quad (3)$$

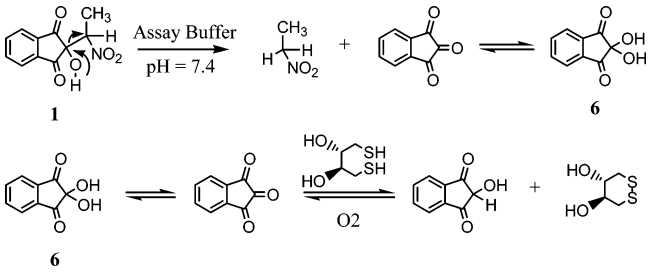
RESULTS

Mechanism of Inhibition of Indandiones. During our screening of HCV NS3 protease inhibitors, we identified several indandiones from our compound library as a novel class of small molecule inhibitors of HCV NS3 protease. Some of the indandiones displayed time-dependent inhibition, while for others the inhibition was established immediately. Compound **1** in Scheme 1 exemplifies the indandiones that showed time-dependent inhibition. Further testing of related compounds **2**−**5** indicates that three functional groups are required for inhibitory activity. The required functional groups include the two ketones on the indane ring, a hydroxyl group on the α-methylene carbon, and a nitro group on the β-carbon. Modification of any one of these functional groups yields mostly inactive compounds except for the case of compound **6**, also known as ninhydrin. The structure of compound **1** and the requirement for all three functional groups for inhibitory activity suggests that compound **1** may undergo a retro-Henry reaction to form ninhydrin as illustrated in Scheme 2. Further examination of the reaction mixture in the presence of these compounds by HPLC

Scheme 1: Structures of Selected Indandiones and their Inhibition Activities (k_{inact}/K_i) against HCV NS3 Protease



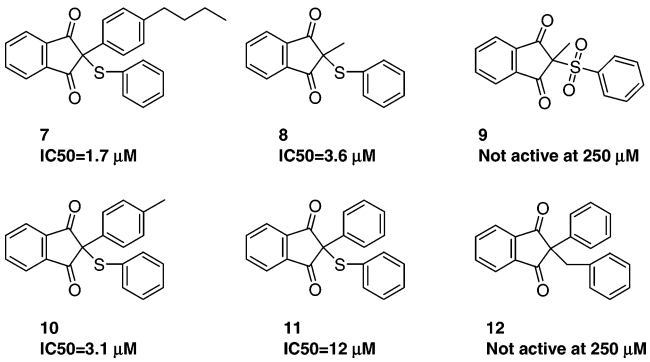
Scheme 2: Breakdown of Selected Indandiones to Ninhydrin by Retro-Henry Reaction and the Subsequent Redox Cycle Involve Ninhydrin, DTT, and the Dissolved Oxygen



revealed the disappearance of dithiothreitol, a reducing reagent that has been shown to be essential in maintaining the optimum enzyme activity and keeping the substrate A P1 cysteine in the reduced form. HPLC also showed a small conversion of the substrate from the reduced form to the oxidized dimer form. Literature precedent indicates that ninhydrin can be reduced to hydroxyninhydrin by the DTT in the assay, and then the dissolved oxygen can reoxidize the hydroxyninhydrin to ninhydrin (*35*). Therefore, ninhydrin, DTT, and oxygen form a redox cycle and slowly consume the DTT in the assay and lead to the formation of the oxidized dimeric disulfide substrate. While the substrate conversion to the dimeric disulfide substrate is less than 5%, a full inhibition was observed at micromolar indandione concentration. This full inhibition cannot be explained by a small reduction in substrate concentration unless the dimeric disulfide substrate itself is a potent inhibitor of HCV NS3 protease. Indeed, when the dimeric disulfide substrate was isolated and tested against substrate B, it displayed slow-binding inhibition of HCV NS3 protease with a K_i value less than 170 nM.

While the previous class of indandione compounds displays time-dependent inhibition and demonstrates a requirement for at least three functional groups for inhibitory activity, the second class of indandiones inhibits HCV NS3 protease immediately at low micromolar concentrations. From the structure and activity analysis of compounds **7**−**12** in Scheme 3, we observed that indandiones that have methylene or sulfonyl linkers to the indandione core showed no inhibitory activity in the assay whereas the *S*-phenyl linked indandiones did inhibit HCV protease. Concerned that the inhibitory activity might arise from the chemical reactivity of the compound and not from binding to the protein, we once again analyzed the reaction mixtures by HPLC. In

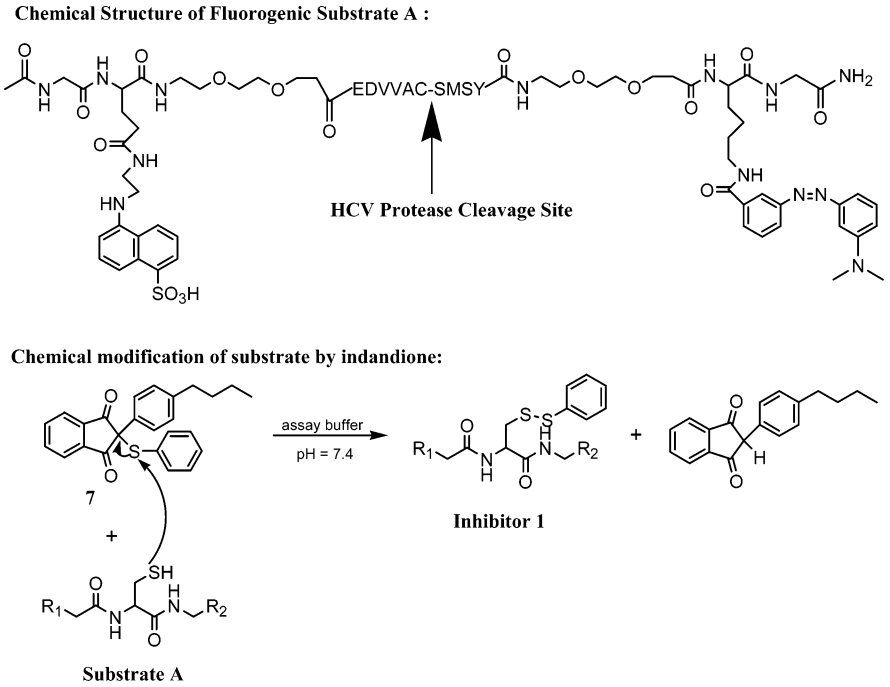
Scheme 3: Structures of Selected Indandiones and their 50% Inhibitory Concentrations (IC_{50}) against HCV NS3 Protease



contrast to the first class of indandione compounds, this class of indandione compounds does not change the DTT concentration; therefore, they do not inhibit HCV NS3 protease by the mechanism established for the first class of indandione compounds. UV monitoring of the DABCYL group absorbance at a wavelength of 512 nm revealed a minor new peak that eluted later than the substrate A. It is interesting that while greater than 90% inhibition can be achieved at 50 μM compound **7**, only about 5% of the substrate is converted to this later eluting peak. Therefore, this class of indandione compounds certainly does not appear to inhibit the HCV NS3 protease activity by reducing the amount of substrate available to the enzyme but instead likely generates a much more potent inhibitor in situ. We characterized the inhibition mechanism of this in situ-formed inhibitor 1. In principle, this later eluting peak could have resulted from a chemical modification of the substrate. The parent substrate structure and the possible chemical modification of it by indandione compounds are illustrated in Scheme 4. In the case of thioether-linked indandiones, the thioether could be attacked by the thiolate of the P1 cysteine residue in the substrate to form a disulfide bond between the substrate and thiophenol. To prove this assumption, we isolated this later eluting peak by preparative HPLC and confirmed the molecular weight of the proposed structure by MALDI/MS as being consistent with inhibitor 1.

Inhibition Constant from the Progress Curve Method. Biphasic reaction progress curves were observed during the inhibition of various of HCV NS3 protease constructs by *S*-phenyl substrate adduct inhibitor 1 (Figure 1), which indicate that this compound is a slow-binding inhibitor of HCV NS3 protease. To investigate the kinetic mechanism of inhibition of HCV NS3 protease by this *S*-phenyl substrate adduct inhibitor 1, we analyzed progress curves obtained using several concentrations of inhibitor. The typical family of plots is illustrated in Figure 1. These curves were fit to eq 1 by nonlinear least-squares regression using Kaleidagraph and yielded values for the three empirical parameters V_0, V_s, and k_{obs}. Reaction velocities at time zero were found to be independent of inhibitor concentration and suggest that any complex that forms prior to the slow step of k_{on} has a dissociation constant much greater than the highest inhibitor concentration tested and thus does not accumulate to any significant concentration. This is supported by the linear dependence of k_{obs} versus inhibitor concentration in Figure 2, which indicates a one-step reaction mechanism (*36*). The

Scheme 4: Structure of Substrate A and the Likely Route of Formation of the *S*-Phenyl Substrate Adduct from Indandiones and Substrate A



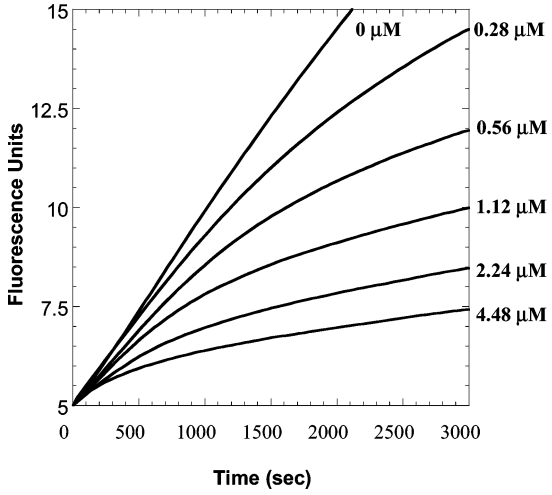


FIGURE 1: Progress curves of 5 μM substrate B cleavage as catalyzed by GST-ΔNS3−NS4A at 0.072−1.44 μM concentrations of *S*-phenyl substrate adduct inhibitor 1.

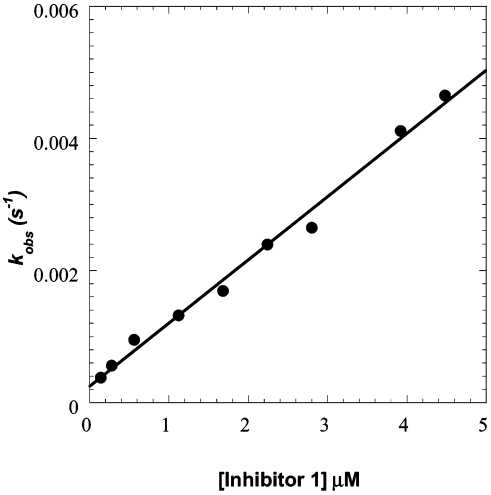


FIGURE 2: Measurement of the k_{on} value of 2370 M^{-1} s^{-1} and k_{off} value of 2.5 × 10^{-4} s^{-1} for *S*-phenyl substrate adduct inhibitor 1 at 5 μM concentration of substrate B.

slope of the curve from Figure 2 times the factor of (1 + [S]/K_m) yielded a k_{on} value of 2370 M^{-1} s^{-1}, and the *y* axis intercept provided an estimate for k_{off} of 2.5 × 10^{-4} s^{-1}. The K_i value calculated from the ratio of those two parameters is 0.10 μM for GST-ΔNS3-NS4A HCV protease. A dilution experiment was performed, where 100 nM of enzyme and 10 × IC_{50} concentration of inhibitor 1 were preincubated for 1 h and then diluted 100-fold into the assay with substrate; the recovery of enzyme activity indicates that inhibitor 1 is a reversible inhibitor (data not shown). However, an attempt to measure the k_{off} rate by using a dilution experiment was hampered by the technical difficulty that the enzyme loses activity significantly in the absence of DTT, but adding DTT to the experiment is not feasible because DTT reduces the disulfide bond of inhibitor. While inhibitor 1 displayed slow-binding kinetics against HCV NS3 protease, its inhibition of human elastase and chymotrypsin were established immediately and required much higher inhibitor concentrations with IC_{50} values of 4 μM for human elastase and 9 μM for chymotrypsin.

Inhibition Constant from the Preincubation Method. The dimeric disulfide substrate and inhibitor 1 are slow-binding inhibitors because, once preincubated with enzyme for 45 min, a linear reaction progress curve could be obtained. The inhibition constant K_i could also be measured by a preincubation method. In the preincubation method, GST-ΔNS3-NS4A HCV protease was incubated with different inhibitor concentrations for 45 min, and then the reactions were started by the addition of substrate. The percent inhibition was calculated from the initial rates of the inhibited reactions relative to the uninhibited control. IC_{50} values were calculated by fitting percent inhibition at six to 12 inhibitor concentrations to eq 2 as shown in Figure 3. The IC_{50} values have also been confirmed by an HPLC analysis method to rule

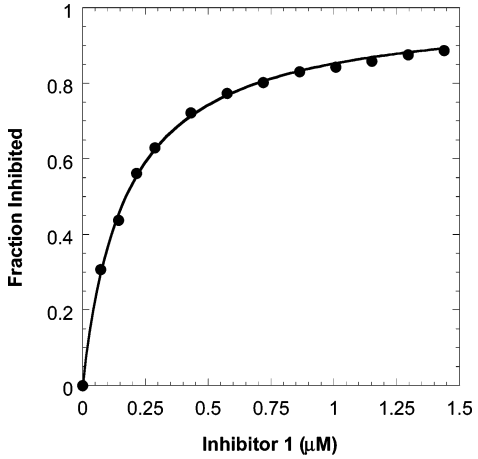


FIGURE 3: Measurement of the K_i value of 0.11 uM for inhibitor 1 by preincubation of various concentrations of inhibitor (0.072−1.44 μM) with 1 nM HCV protease and assayed at a substrate concentration of 0.5 K_m.

out false inhibition by spectral artifacts. K_i values of 0.17 and 0.11 μM were calculated from eq 3 for the dimeric disulfide substrate and inhibitor 1 assuming competitive inhibition (*37*). This value is in good agreement with that obtained from the progress curve method, thereby confirming the accuracy of both k_{on} and k_{off} measured by the slow-binding progress curve method.

Origin of the Slow Binding. Slow binding is a complex phenomenon in enzymology, and its underlying mechanism varies from case to case (*33*). However, establishing the origin of slow-binding inhibition can sometimes provide insight toward the inhibition mechanism. We considered two possible explanations for the slow binding of inhibitor 1. This compound contains a chemically labile disulfide bond, and so it is possible that inhibitor 1 could be slowly reacting with residues on the enzyme in a reversible process. A second possible explanation for the slow binding is that a slow enzyme conformational change may be occurring to accommodate the larger P1 substituent group of inhibitor 1 relative to the P1 of the substrate. To test both hypotheses, we synthesized a stable analogue of inhibitor 1 in which the S−SPh group was changed to a S−CH_2Ph group. The P1 side chain of this analogue is similar in size to that of the inhibitor 1 but does not have the chemically labile properties of a sulfur−sulfur bond. When this analogue was tested with 45 min preincubation and even at 40 μM concentration, it did not show any inhibition of HCV NS3 protease activity. This result argues against the enzyme conformational change mechanism being responsible for the slow binding of inhibitor 1. However, this result supports the view that the slow binding of inhibitor 1 may arise from the chemical lability of the disulfide bond.

The mechanism of the chemically labile nature of the disulfide compound probably involves the formation of mixed disulfides between the compound and one or more of the protein cysteines. Precedent for such an inhibition mechanism derives from studies of the inhibition of the serine protease from human cytomegalovirus (HCMV) protease by a symmetrically substituted disulfide compound (CL13933). CL13933 forms covalent disulfide adducts with HCMV protease (*38*). It was proposed that formation of a disulfide adduct with a specific Cys residue near the active site locks

Table 1: K_i of *S*-Phenyl Substrate Adduct Inhibitor 1 against Various Enzyme Constructs Was Obtained from Preincubation of Various Concentrations of Inhibitor (0.072−1.44 μM) with 1 nM HCV Protease and Assayed at a Substrate A Concentration of 0.5 K_m[a]

construct	K_m (μM)	K_i (μM)	-fold difference
GST-$NS3_{1-181}$-$NS4A_{54}$	3.4	0.11	31
$NS3_{1-181}$ + $NS4A_{21-39}$(wt)	11.3	0.06	190
$NS3_{1-181}$ + $NS4A_{21-39}$(C159S)	9.3	0.14	72

[a] K_m of substrate A was obtained from the Michaelis−Menten equation using the inner-filter effect corrected initial rates at substrate concentrations from 0.25 to 5 K_m values.

HCMV protease in an inactive conformation, resulting in inhibition. To further investigate potential enzyme residues involved in disulfide exchange with the inhibitor 1, we examined the HCV NS3 protease structure and noted that the Cys-159 residue is 10 Å away from the active site serine 139. We then created the cysteine 139 to serine mutant. Both wild-type and mutant enzyme were kinetically characterized, and the inhibition of inhibitor 1 against both enzymes was compared.

We have examined the K_m and k_{cat}/K_m for wild-type and C159S mutant HCV NS3 protease against fluorogenic substrates using the plate reader fluorogenic assay. The inner filter effect becomes important when conducting enzyme kinetic experiments using multiple concentrations of fluorogenic substrate (*39*). Therefore, the inner filter effect has been corrected in the measurement of our K_m and k_{cat}/K_m values for wild-type and C159S mutant HCV NS3 proteases. We obtained a K_m value of 1.0 μM and k_{cat}/K_m of 115 000 M^{-1} s^{-1} for wild-type HCV NS3 protease and a K_m of 1.0 μM and k_{cat}/K_m of 76 000 M^{-1} s^{-1} for C159S mutant enzyme using the 4A/4B depsipeptide substrate B. Likewise, the fluorogenic 5A/5B amide substrate A exhibited K_m values of 11.3 and 9.3 μM for wild-type and C159S mutant enzyme, respectively. These results indicate that the wild-type and C159S mutant enzyme exhibit nearly identical catalytic properties.

The K_i values for the inhibitor 1, together with K_m values of its parent substrate against various enzyme constructs, are shown in Table 1. There is only a 2-fold difference in the K_i value of the inhibitor 1 against wild-type and C159S mutant enzymes. This result would argue against an important role for Cys-159 in the inhibition mechanism.

Although Cys-159 is the nearest to the active site serine among all seven cysteine residues in the NS3 protease domain, our above work has shown it does not have a direct effect on HCV NS3 protease catalyzed hydrolysis. Mutation analysis by Hijikata et al. (*40*) suggested that, in contrast to Cys-16, Cys-47, Cys-52, and Cys-159, which appear to be dispensable, Cys-97, Cys-99, and Cys-145 are critical for HCV NS3 protease activity and are involved in structural zinc chelating. The possible oxidization of any of enzyme cysteines by inhibitor 1 or the dimeric disulfide substrate would lead to distorted structural zinc binding and inhibition of protease activity. Because the Cys-97, Cys-99, and Cys-145 mutant enzymes lose all of their catalytic activity (*40*), it is not possible to test inhibitor 1 against these mutant enzymes.

DISCUSSION

We have found that certain indandiones are apparent inhibitors of HCV NS3 protease. In one class, the apparent inhibition is due to the redox chemistry of the compound, and in the other the inhibition is due to the chemical instability of the compounds. However, the true inhibitors, either the oxidized dimeric disulfide substrate formed because of the consumption of the reducing agent DTT by the first class of indandiones or the *S*-phenyl substrate adduct inhibitor 1, formed from the attack of the substrate A P1 cysteine side chain on the *S*-phenyl linked indandiones, inhibits the enzyme in a time-dependent manner to ultimately form an enzyme inhibitor complex with a K_i value in the 100 nM range.

We investigated two possible inhibitory mechanisms that might explain the slow binding of these inhibitors to the HCV NS3 protease. One proposed mechanism suggests that the slow binding results from an enzyme conformational change possibly required to accommodate the large P1 substituents present in these two inhibitors. It is quite interesting that formation of a disulfide adduct, even as small as an *S*-phenyl, converts a good substrate into a potent inhibitor. Various substrate specificity studies indicate that the P1 pocket prefers small, hydrophobic groups such as Cys, Thr, and Abu (*41*, *42*). The Phe-154 side chain located at the bottom of the pocket defines the size of the hydrophobic P1 pocket. The sulfhydryl−aromatic interaction was earlier proposed as a substrate recognition mechanism (*41*). In the absence of structural information, we speculate that the slow on rate that we measured might arise from a slow enzyme conformational change. It is likely that an enzyme conformational change might be necessary to accommodate this *S*-phenyl group of inhibitor 1 into the P1 pocket that has so far been defined as small and hydrophobic. If an enzymatic conformational change did occur then it might disrupt the active site architecture so that the bound peptide ligand could not be cleaved, thereby accounting for the inhibition. However, our testing of the stable analogue with the benzyl group linked to the P1 sulfhydryl established that this compound was neither a substrate nor an inhibitor of NS3 protease activity at concentrations as high as 40 μM. This result confirms the P1 specificity for small, hydrophobic groups and argues against a possible enzyme conformational change as the mechanism responsible for the slow binding of inhibitor 1 or the dimeric disulfide substrate. This result also suggests that substrate-like peptides with bulky P1 side chains do not bind well to the substrate binding site. Therefore, it seems likely that inhibitor 1 and the dimeric disulfide substrate exert their potent inhibition of NS3 protease via interactions that are outside of the substrate binding site.

The other inhibition mechanism that could possibly account for the slow binding involves disulfide exchange between the inhibitor 1 or the dimeric disulfide substrate and the sulfhydryl group of the Cys-159 residue, thereby generating an inhibited, covalently modified enzyme. Precedent for such an inhibition mechanism derives from studies of the inhibition of the serine protease from human cytomegalovirus (HCMV) protease by a symmetrically substituted disulfide compound (CL13933). CL13933 forms covalent disulfide adducts with HCMV protease (*38*). It was proposed that formation of a disulfide adduct with a specific Cys residue near the active site locks HCMV protease in an inactive conformation, resulting in inhibition. In principle, a similar mechanism could be proposed for inhibitor formation with the Cys 159 side chain of HCV NS3 protease with the disulfide inhibitor 1. However, we have shown that the Cys159Ser NS3 protease, which cannot undergo disulfide exchange with the inhibitor 1, also undergoes time-dependent inhibition by the inhibitor 1. Therefore, an inhibitory mechanism involving disulfide adduct formation with the Cys159 can be ruled out.

We have excluded slow-binding mechanisms of inhibition by inhibitor 1 involving an enzyme conformational change or a chemical modification of the enzyme by disulfide exchange with the Cys159. However, the slow-binding phenomenon associated with the inhibitor 1 or the dimeric disulfide substrate could be reflecting a sulfhydryl group transfer from the inhibitor to any of the conserved cysteines involved in chelation of the structural zinc resulting in distortion of the protease structure. Other small disulfide compounds have previously been described to inhibit the HCV NS3 protease by acting as Zn^{2+}-ejecting reagents (*43*). Compounds of this type were previously shown to be able to eject zinc from the HIV nucleocapsid protein (*44*) or block the maturation of Murine Leukemia virus by disulfide cross-linking (*45*). Inhibitor 1 or the dimeric disulfide substrate might behave similarly to these small disulfide compounds and form disulfides with any of the three cysteines involved in zinc chelation and thereby distort the native coordination sphere.

Targeting the structure zinc chelating is not novel to the HCV NS3 protease drug discovery. Many chelators such as phenanthrolines have been shown to inhibit HCV NS3 protease activity. Although the chemically labile nature of the disulfide bond will likely prevent inhibitor 1 from becoming a drug development candidate, the implication of its inhibition mechanism sheds some light on future drug discovery targeting HCV NS3 protease. Despite the chemically labile nature of disulfide compounds, we note that patent disclosures on compounds comprising disulfide-containing peptides for treatment and prevention of infectious diseases such as viral hepatitis B and C, AIDS, and herpes continue to emerge (*46*, *47*).

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