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J. Med. Chem., Just Accepted Manuscript • DOI: 10.1021/acs.jmedchem.7b00497 • Publication Date (Web): 03 Jul 2017

Downloaded from http://pubs.acs.org on July 4, 2017

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ABSTRACT

Ester and carbamate prodrugs of aldehyde bisulfite adduct inhibitors were synthesized in order to improve their pharmacokinetic and pharmacodynamic properties. The inhibitory activity of the compounds against norovirus 3C-like protease in enzyme and cell-based assays was determined. The ester and carbamate prodrugs displayed equivalent potency to those of the precursor aldehyde bisulfite adducts and precursor aldehydes. Furthermore, the rate of ester cleavage was found to be dependent on alkyl chain length. The generated prodrugs exhibited low cytotoxicity and satisfactory liver microsomes stability and plasma protein binding. The methodology described herein has wide applicability and can be extended to the bisulfite adducts of common warheads employed in the design of transition state inhibitors of serine and cysteine proteases of medical relevance.
Introduction

Human noroviruses are the principal cause of non-bacterial acute gastroenteritis worldwide, consequently, they have a major impact on public health.\textsuperscript{1-5} It is estimated that noroviruses are responsible for 19-21 million infections, 56,000-71,000 hospitalizations, and 700-800 deaths annually in the USA.\textsuperscript{6-7} Morbidity is particularly high among the young and elderly, as well as immunocompromised individuals.\textsuperscript{8} In developing countries, the mortality rate among children <5 years old due to diarrheal disease caused by noroviruses is estimated to account for 71,000 deaths annually.\textsuperscript{9-11} The problem is further exacerbated by the high infectivity, genetic diversity, copious virus shedding, and environmental stability of noroviruses.\textsuperscript{12-13} Other factors that compound the problem and hamper drug discovery efforts include the lack of a robust animal model that recapitulates all aspects of the disease\textsuperscript{14-15} and an incomplete understanding of norovirus biology and pathogenesis.\textsuperscript{16-20} Collectively, the management of norovirus infections presents a challenge because no effective vaccines or norovirus-specific therapeutics or prophylactics are currently available\textsuperscript{21-25}.

As part of an ongoing research program focused on the discovery of antiviral therapeutics and prophylactics for norovirus and picornaviral infections,\textsuperscript{26-29} we have recently reported the structure-guided design, synthesis, and biochemical evaluation of peptidyl aldehydes,\textsuperscript{30} α-ketoamides,\textsuperscript{31} and their corresponding bisulfite adducts,\textsuperscript{32} as inhibitors of norovirus 3C-like protease (3CLpro) and related picornaviral 3C protease and coronavirus 3CLpro. These inhibitors were found to potently inhibit norovirus in a cell-based replicon system and to exhibit efficacy in a small animal model of norovirus
Importantly, the generated bisulfite adducts were found to display pharmacological activity in enzyme and in a cell-based replicon system comparable to the precursor aldehydes and α-ketoamides. We have furthermore recently demonstrated for the first time that a broad-spectrum peptidyl aldehyde bisulfite adduct coronavirus protease inhibitor reverses infection progression of fatal coronavirus infection in cats caused by feline infectious peritonitis virus (FIPV), when administered subcutaneously. In order to further optimize the PK characteristics of the bisulfite adducts and identify an orally-bioavailable drug candidate, we report herein the design, synthesis and preliminary evaluation of novel ester (I) and carbamate (II) prodrug forms (Figure 1) of transition state (TS) inhibitor bisulfite adducts as a general strategy for improving the physicochemical properties and PK characteristics of carbonyl-containing TS inhibitors of serine and cysteine proteases.

Results and Discussion

Design rationale. Peptidyl and non-peptidyl aldehydes, α-ketoamides, α-ketoheterocycles, α-ketoesters and others, are frequently employed as transition state inhibitors of mammalian, viral, and parasitic serine and cysteine proteases of clinical relevance, however, the therapeutic potential of these inhibitors, particularly aldehyde-based inhibitors, is adversely impacted by their sub-optimal pharmacokinetics and low oral bioavailability. These considerations provided the impetus for conducting further studies to identify structural variants that display improved physicochemical properties and are likely to be orally-bioavailable. Thus, we initially explored the utilization of bisulfite adducts as a latent form of the warheads used in TS inhibitors. In order to further optimize the PK characteristics of the aldehyde bisulfite adducts, it was...
envisaged that further structural modifications would likely result in additional improvements in the physicochemical and PK characteristics of these compounds. Specifically, the hydroxyl group in bisulfite adducts provided a convenient site for further derivatization to yield ester (I) and carbamate (II) prodrugs (Figure 1). Thus, the design and mechanism of action of prodrugs (I-II), entails enzyme-mediated or non-enzymatic hydrolysis that converts prodrugs (I-II) into the aldehyde bisulfite adduct which subsequently reverts to the precursor aldehyde (Figure 2). Although this has not been demonstrated as yet, it is possible that the aldehyde bisulfite adduct itself may have intrinsic inhibitory activity toward NV 3CLpro by functioning as a transition state mimic. Subsequent reaction with the catalytic cysteine (Cys139) of norovirus 3CLpro leads to inactivation of the enzyme via the formation of a tetrahedral adduct (Figure 3). The increased lipophilicity of ester prodrugs (I) was anticipated to result in enhanced absorption, and the rate and extent of absorption of (I) can be conveniently modulated by varying the hydrophobic component (R). Furthermore, the rate of non-enzymatic activation of prodrugs (I) can also be conveniently controlled through the use of appropriate esters and strategies. Lastly, carbamate prodrugs (II) derived from amino acids offer additional advantages, including control of the hydrolysis rate via the use of natural and unnatural amino acids.

**Chemistry.** Prodrugs (I-II) were readily synthesized by refluxing the peptidyl aldehyde bisulfite adduct with an appropriate anhydride, chloroformate or amino acid-derived isocyanate in dry acetonitrile (Schemes 1 and 2) and are listed in Tables 1 and 2.

**Biochemical Studies.** The inhibitory activity of the synthesized compounds against NV 3CLpro and their anti-norovirus activity in a cell-based replicon system were evaluated.
as described in the experimental section.\textsuperscript{26-27} The determined IC\textsubscript{50} values in enzyme assay and EC\textsubscript{50} values against NV in replicon harboring cells (HG23 cells) are listed in Tables 1 and 2 and they are the average of at least two determinations.

The results listed in Table 1 clearly demonstrate that prodrugs \textbf{1-17} display significant anti-norovirus activity in replicon cells. Furthermore, these compounds were found to exhibit comparable inhibitory activity against norovirus 3CLpro, as well as norovirus in replicon cells, as the precursor bisulfite adducts and corresponding aldehydes. As anticipated, the \textit{m}-Cl phenyl substituted derivatives with a cyclohexylalanine (Cha) as the P\textsubscript{2}\textsuperscript{51} residue (compounds \textbf{9-14}, Table 1), displayed higher potency because the Cha side chain optimally fills the hydrophobic S\textsubscript{2} subsite and the chlorine substituent engages in additional binding interactions.\textsuperscript{26} The prime subsites (S\textsubscript{n}') of norovirus 3CLpro are shallow, consequently, prodrugs \textbf{1-17} are presumed to be devoid of intrinsic inhibitory activity toward 3CLpro or norovirus, however, under the conditions used to assay the enzyme or in conducting the replicon harboring cells studies, hydrolysis of the esters yields the corresponding TS inhibitor bisulfite adducts which then revert to the corresponding aldehydes, the putative inhibitory species. This hypothesis is supported by the results of structural studies where incubation of a peptidyl aldehyde bisulfite adduct with norovirus 3CLpro 20 mM Tris buffer, pH 8.0, resulted in reversal of the bisulfite adduct to the precursor aldehyde, followed by reaction with the active site cysteine (Cys139), ultimately leading to the formation of an enzyme-aldehyde inhibitor adduct. The high-resolution structure of the enzyme-inhibitor complex provides cogent confirmation of this hypothesis.\textsuperscript{26} Furthermore, utilization of prodrug \textbf{9} in the cell-based
assay, followed by mass spectrometric analysis, has established the formation of aldehyde 25.

A small number of carbamate prodrugs 18-23 (Table 2) were also synthesized and shown to exhibit potent anti-norovirus activity (Table 2, compounds 21-22). The availability of a large number of natural and unnatural amino acids makes possible the fine tuning of the rate and extent of absorption, as well as release in blood plasma, of the inhibitory species. Likewise, the high potency and lower chemical reactivity of carbonate esters can also be exploited (for example, compound 17 in Table 1).

**Hydrolysis of ester prodrugs to the aldehyde in mouse and human serum.** The hydrolysis of the bisulfite adducts and three prodrug compounds in mouse and human serum was initially determined by incubating the compounds at 37 °C for 1 h and the results are summarized in Table 3. At the end of the incubation period, most of the bisulfite adducts forms of compounds 24 and 26 were converted to the active aldehyde counterparts in the presence of mouse or human serum. For the prodrug compounds, the hydrolysis of compounds 9 and 10 was shown to be species specific: complete conversion to the aldehyde in mouse serum versus <50% conversion human serum in 1 h. To determine detailed kinetics of hydrolysis of the precursor ester prodrugs, compounds 9, 10, and 11, were incubated separately in 55% human serum, aliquots were withdrawn at different time intervals, and the % remaining ester prodrug was plotted over time (Figure 4). Esterified derivatives 9 and 10 were found to release the putative inhibitory species (aldehyde) rapidly (half-life ~25 minutes), while the hydrolysis of the n-hexanoic acid-derived ester 11 was much slower. These results demonstrate that ester prodrugs (I) are capable of releasing the active inhibitory species in blood.
serum and that the nature of the R group can be used to modulate the rate of release. The hydrolysis of the ester prodrugs could, in principle, proceed through a spontaneous or esterase-mediated hydrolysis (or both).

**Cytotoxicity.** The potential toxicity to human HeLa cells was determined using a representative set of prodrugs and precursor compounds and the results are summarized Table 4. The prodrugs, as well as the precursor aldehyde and aldehyde bisulfite adducts were all relatively nontoxic in this assay, with \( CC_{50} \) values ranging from 50 to >200 µM. The selectivity index was calculated as the ratio between toxicity and potency \( (CC_{50} \text{ HeLa}/EC_{50}) \) and the values ranged from >667 to >6,667, demonstrating an excellent selectivity for the viral target.

**Liver microsomes stability.** The stability of a select number of compounds in liver microsomes was determined. The stability of the aldehyde form of 24 (Figure 5) was high when exposed to mouse and human liver microsomes (Table 5), a desirable property for an active compound. The chloro-substituted analogs, 25 and 26, were both present in solution as the aldehyde form prior to the addition of microsomes. As expected, both compounds behaved similarly (40 and 35% remaining, respectively) in the presence of mouse microsomes. In the presence of mouse liver microsomes prodrug 11 was converted to the aldehyde form 25 (Figure 5) and to other forms, with 38% remaining after 1 h. Specifically, LC-MS was used to establish the presence of aldehyde 25, as well as the presence of a reduced alcohol form. Importantly, compound 11 was only moderately metabolized (76% remaining) by human liver microsomes after 1 h.
Plasma protein binding. Binding to albumin, α1-glycoprotein and red blood cells impacts drug distribution, PK parameters and exposure to the target. Consequently, the extent of binding to plasma proteins of a select number of compounds was also investigated. Compounds were tested for their ability to bind to plasma proteins over a 4-h time period. Chloro-substituted analogs 11, 25, and 26 were strongly bound to mouse and human serum (Table 6), whereas the aldehyde form of 24 demonstrated only moderate binding (65-71%). Since binding to albumin and α1-glycoprotein impacts drug distribution and exposure to the target, the moderate plasma protein binding of compound 24 is considered favorable from a drug development perspective.

Conclusions

A general approach toward the optimization of the pharmacokinetics of peptidyl and non-peptidyl transition state inhibitors via the synthesis of ester and carbamate prodrugs of aldehyde bisulfite adducts is reported. The ester prodrugs were found to undergo hydrolysis in blood plasma with a half-life of ~0.25 h. The rate of hydrolysis was dependent on the ester alkyl chain. The generated prodrugs also exhibited low cytotoxicity and satisfactory liver microsomes stability and plasma protein binding. The approach has wide applicability and provides an effective means of augmenting the therapeutic potential of these classes of inhibitors. It should be noted that the prodrug approach expounded herein can be extended to other transition state inhibitors, such as α-ketoamides. Lastly, previously reported peptidyl or nonpeptidyl TS inhibitors of mammalian, viral, and parasitic serine and cysteine proteases such as, for example, cathepsins L and S, calpains, and falcipain-2, can all be transformed to the
corresponding ester and carbamate prodrugs. We have indeed demonstrated this using known cathepsin L, falcipain-2, and calpain inhibitors (unpublished results).

**Experimental section**

**General.** Reagents and dry solvents were purchased from various chemical suppliers (Aldrich, Acros Organics, Chem-Impex, TCI America, Oakwood chemicals, Bachem, and Fisher) and were used as obtained. Silica gel (230-450 mesh) used for flash chromatography was purchased from Sorbent Technologies (Atlanta, GA). The $^1$H spectra were recorded in CDCl$_3$ or DMSO-d$_6$ on a Varian XL-400 NMR spectrometer. High resolution mass spectra (HRMS) were performed at the University of Kansas Mass Spectrometry lab using an LCT Premier mass spectrometer (Waters, Milford, MA) equipped with a time of flight mass analyzer and an electrospray ion source. Thin layer chromatography was performed using Analtech silica gel plates and visualization was accomplished using UV light and/or iodine. The purity of the compounds was determined by high-performance liquid chromatography (HPLC) using a Varian Pro-star HPLC system with a normal phase column (Kinetex 2.6u HILIC 100A, 75 x 4.6mm) at 254 nm. Analysis was conducted using two different methods. Method A: isocratic with 40% acetonitrile and 60% dichloromethane, mobile phase flow rate 1.0 mL/min. Method B: isocratic with 20% acetonitrile and 80% dichloromethane, mobile phase flow rate 1.0 mL/min. All final compounds had a purity of ≥95% by both methods.

**Synthesis of compounds 1-15: General Procedure.** Alkanoic anhydride (1 mmol) was added to a solution of dipeptidyl bisulfite salt (1 mmol) in dry acetonitrile 10 mL and the reaction mixture was refluxed for 2 h with stirring. The solvent was removed in
vacuo. The residue was washed thoroughly with ethyl ether until a white precipitate formed. The supernatant was carefully removed using pipette and the white precipitate was dried *in vacuo*.

(5S,8S)-5-Isobutyl-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-1-phenyl-2,10-dioxa-4,7-diazadodecane-9-sulfonate (1). Yield (67 %), mp 94-97 °C. $^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm 7.86 - 7.93 (m, 1H), 7.62 - 7.71 (m, 1H), 7.48 (br. s., 1H), 7.23 - 7.40 (m, 5H), 5.30 (d, $J = 2.34$ Hz, 1H), 4.96 - 5.07 (m, 2H), 4.08 - 4.20 (m, 1H), 3.93 (d, $J = 8.30$ Hz, 1H), 3.09 (br. s., 2H), 2.05 - 2.20 (m, 1H), 2.02 (s, 3H), 1.87 - 1.95 (m, 2H), 1.66 - 1.73 (m, 1H), 1.51 - 1.64 (m, 2H), 1.27 - 1.49 (m, 2H), 0.79 - 0.88 (m, 6H). HRMS (ESI) calcd for C$_{23}$H$_{32}$N$_3$O$_9$S: [M$^+$]: 526.1865. Found: 526.1857. HPLC purity 96.4% (Method A).

(5S,8S)-5-Isobutyl-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-1-phenyl-2,10-dioxa-4,7-diazatridecane-9-sulfonate (2). Yield (63 %), mp 83-85 °C. $^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm 7.83 - 7.91 (m, 1H), 7.55 (t, $J = 7.62$ Hz, 1H), 7.45 - 7.51 (m, 1H), 7.35 (d, $J = 4.30$ Hz, 5H), 5.58 - 5.67 (m, 1H), 4.93 - 5.08 (m, 2H), 4.14 - 4.28 (m, 1H), 3.90 - 4.12 (m, 1H), 2.93 - 3.19 (m, 2H), 2.00 - 2.26 (m, 2H), 1.83 - 1.96 (m, 1H), 1.74 - 1.82 (m, 2H), 1.54 - 1.71 (m, 4H), 1.31 - 1.52 (m, 1H), 0.95 (t, $J = 7.42$ Hz, 3H), 0.75 - 0.90 (m, 6H). HRMS (ESI) calcd for C$_{24}$H$_{34}$N$_3$O$_9$S: [M$^+$]: 540.2021. Found: 540.2013. HPLC purity 95.8% (Method A).

(5S,8S)-5-Isobutyl-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-1-phenyl-2,10-dioxa-4,7-diazatetradecane-9-sulfonate (3): Yield (68 %), mp 77-79 °C. $^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm 8.60 - 8.66 (m, 1H), 8.49 - 8.55 (m, 1H), 7.62 - 7.71 (m, 1H), 7.26 - 7.57 (m, 5H), 5.60 - 5.68 (m, 1H), 4.94 - 5.18 (m, 2H), 3.83 - 4.05 (m, 1H), 3.74 (d,
\[
J = 7.03 \text{ Hz}, 1H), 2.98 - 3.18 (m, 2H), 2.12 (br. s., 2H), 1.99 (br. s., 1H), 1.60 (br. s., 3H), \\
1.46 (d, J = 7.23 \text{ Hz}, 6H), 1.11 (t, J = 6.74 \text{ Hz}, 3H), 0.85 (br. s., 6H). HRMS (ESI) calcd \\
for C_{25}H_{36}N_{3}O_{9}S: [M-]: 554.2178. Found: 554.2166. HPLC purity 98.2\% (Method A).
\]

(5S,8S)-5-Isobutyl-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-1-phenyl-2,10-dioxa-4,7-diazapentadecane-9-sulfonate (4): Yield (52 \%), mp 80-83 \degree C. \textsuperscript{1}H NMR \\
(400 MHz, DMSO-d_{6}) \delta ppm 8.45 - 8.51 (m, 1H), 7.61 - 7.66 (m, 1H), 7.44 - 7.53 (m, \\
1H), 7.35 (br. s., 5H), 5.10 - 5.15 (m, 1H), 4.98 - 5.07 (m, 2H), 4.17 - 4.26 (m, 1H), 4.03 \\
- 4.13 (m, 1H), 3.00 - 3.17 (m, 2H), 2.16 - 2.22 (m, 2H), 1.86 - 1.94 (br. s., 1H), 1.60 - \\
1.70 (m, 2H), 1.43 - 1.54 (m, 4H), 1.23 - 1.35 (m, 5H), 1.10 (t, J = 7.78 \text{ Hz}, 3H), 0.80 - \\
0.91 (m, 6H). HRMS (ESI) calcd for C_{26}H_{38}N_{3}O_{9}S: [M-]: 568.2334. Found: 568.2336. \\
HPLC purity 94.7\% (Method A).

(5S,8S)-5-Isobutyl-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-1-phenyl-2,10-dioxa-4,7-diazahexadecane-9-sulfonate (5): Yield (62 \%), mp 72-75 \degree C. \textsuperscript{1}H NMR \\
(400 MHz, DMSO-d_{6}) \delta ppm 7.58 - 7.68 (m, 1H), 7.50 (d, J = 8.20 \text{ Hz}, 1H), 7.44 (d, J = 3.91 \\
Hz, 1H), 7.28 - 7.40 (m, 5H), 5.06 (s, 1H), 4.99 - 5.04 (m, 2H), 3.91 - 4.00 (m, 1H), 3.81 \\
- 3.85 (m, 1H), 2.97 - 3.15 (m, 2H), 2.07 - 2.20 (m, 2H), 1.87 - 1.91 (m, 1H), 1.57 - 1.64 \\
(m, 2H), 1.40 - 1.49 (m, 7H), 1.28 (br. s., 4H), 1.10 (t, J = 6.20 \text{ Hz}, 3H), 0.80 - 0.91 (m, \\
6H). HRMS (ESI) calcd for C_{27}H_{40}N_{3}O_{9}S: [M-]: 582.2491. Found: 582.2487. HPLC purity \\
97.2\% (Method A).

(5S,8S)-5-Isobutyl-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-1-phenyl-2,10-dioxa-4,7-diazaoctadecane-9-sulfonate (6): Yield (61 \%), mp 74-77 \degree C. \textsuperscript{1}H NMR \\
(400 MHz, DMSO-d_{6}) \delta ppm 8.45 - 8.51 (m, 1H), 7.62 - 7.66 (m, 1H), 7.46 - 7.54 (m, 1H), \\
7.28 - 7.42 (m, 5H), 5.31 - 5.34 (m, 1H), 4.96 - 5.06 (m, 2H), 4.16 - 4.22 (m, 1H), 4.05 -
4.11 (m, 1H), 3.00 - 3.17 (m, 2H), 2.09 - 2.20 (m, 5H), 1.40 - 1.54 (m, 6H), 1.20 - 1.32 (m, 9H), 1.11 (t, J = 6.70 Hz, 3H), 0.86 (br. s., 6H). HRMS (ESI) calcd for C_{29}H_{44}N_{3}O_{9}S: [M-]: 610.2804. Found: 610.2817. HPLC purity 95.8% (Method A).

(5S,8S)-5-Isobutyl-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-1,11-diphenyl-2,10-dioxa-4,7-diazaundecane-9-sulfonate (7): Yield (51 %), mp 84-87 °C. ¹H NMR (400 MHz, DMSO-d$_6$) δ ppm 8.51 - 8.56 (m, 1H), 8.15 (d, J = 7.13 Hz, 1H), 7.93 (d, J = 7.03 Hz, 2H), 7.60 - 7.70 (m, 1H), 7.46 - 7.57 (m, 3H), 7.25 - 7.43 (m, 5H), 5.33 - 5.38 (m, 1H), 4.97 - 5.09 (m, 2H), 4.14 - 4.35 (m, 1H), 3.82 - 4.11 (m, 1H), 2.93 - 3.20 (m, 2H), 1.97 - 2.35 (m, 3H), 1.74 - 1.95 (m, 1H), 1.54 - 1.69 (m, 2H), 1.28 - 1.51 (m, 2H), 0.74 - 0.92 (m, 6H). HRMS (ESI) calcd for C$_{28}$H$_{34}$N$_3$O$_9$S: [M-]: 588.2021. Found: 588.2017. HPLC purity 96.7% (Method A).

(5S,8S)-5-Isobutyl-12-methyl-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-1-phenyl-2,10-dioxa-4,7-diazatridecane-9-sulfonate (8): Yield (69 %), mp 71-73 °C. ¹H NMR (400 MHz, DMSO-d$_6$) δ ppm 8.46 - 8.52 (m, 1H), 7.59 - 7.63 (m, 1H), 7.46 - 7.54 (m, 1H), 7.32 (d, J = 1.56 Hz, 5H), 4.93 - 5.05 (m, 3H), 4.14 - 4.23 (m, 1H), 4.01 - 4.08 (m, 1H), 3.27 - 3.38 (m, 2H), 3.03 - 3.12 (m, 2H), 2.11 - 2.21 (m, 1H), 1.52 - 1.65 (m, 2H), 1.38 - 1.50 (m, 2H), 1.01 - 1.12 (m, 2H), 0.95 (d, J = 6.93 Hz, 6H), 0.84 (d, J = 7.23 Hz, 6H). HRMS (ESI) calcd for C$_{25}$H$_{36}$N$_3$O$_9$S: [M-]: 554.2178. Found: 554.2158. HPLC purity 94.6% (Method A).

(5S,8S)-1-(3-Chlorophenyl)-5-(cyclohexylmethyl)-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-2,10-dioxa-4,7-diazadodecane-9-sulfonate (9): Yield (69 %), mp 94-96 °C. ¹H NMR (400 MHz, DMSO-d$_6$) δ ppm 8.46 - 8.52 (m, 1H), 7.61 - 7.64 (m, 1H), 7.54 - 7.58 (m, 1H), 7.46 - 7.51 (m, 1H), 7.34 - 7.43 (m, 2H), 7.27 - 7.32
(m, 1H), 5.28 - 5.32 (m, 1H), 5.04 (d, J = 5.66 Hz, 2H), 4.14 - 4.22 (m, 1H), 4.06 - 4.13 (m, 1H), 3.01 - 3.20 (m, 2H), 2.22 - 2.36 (m, 1H), 1.99 - 2.19 (m, 4H), 1.97 (s, 3H), 1.54 - 1.74 (m, 6H), 1.49 (br. s., 1H), 1.06 - 1.21 (m, 4H), 0.76 - 0.96 (m, 2H). HRMS (ESI) calcd for C_{26}H_{35}ClN_{3}O_{9}S: [M-]: 600.1788. Found: 600.1719. HPLC purity 98.3% (Method A).

(5S,8S)-1-(3-chlorophenyl)-5-(cyclohexylmethyl)-3,6,11-trioxo-8-((2-oxopyrrolidin-3-yl)methyl)-2,10-dioxa-4,7-diazatridecane-9-sulfonate (10): Yield (75 %), mp 74-77 °C. \(^1\)H NMR (400 MHz, DMSO-d\(_6\)) \(\delta\) ppm 8.47 - 8.52 (m, 1 H), 7.74 - 7.79 (m, 1 H), 7.66 (d, J=8.40 Hz, 1 H), 7.49 (d, J=8.98 Hz, 1 H), 7.33 - 7.45 (m, 2 H), 7.29 (br. s., 1 H), 5.03 (br. s., 2 H), 4.67 (br. s., 1 H), 4.01 (d, J=6.44 Hz, 1 H), 3.68 - 3.79 (m, 1 H), 3.09 (d, J=8.40 Hz, 2 H), 2.99 (d, J=7.81 Hz, 2 H), 2.17 - 2.30 (m, 2 H), 2.10 (br. s., 2 H), 1.74 - 1.84 (m, 1 H), 1.48 - 1.72 (m, 4 H), 1.34 - 1.46 (m, 2 H), 1.30 (d, J=6.05 Hz, 1 H), 0.95 - 1.16 (m, 6 H), ppm 0.85 (br. s., 3 H). HRMS (ESI) calcd for C_{27}H_{37}ClN_{3}O_{9}S: [M-]: 614.1912. Found: 614.1947. HPLC purity 98.2% (Method A).

(5S,8S)-1-(3-Chlorophenyl)-5-(cyclohexylmethyl)-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-2,10-dioxa-4,7-diazahexadecane-9-sulfonate (11): Yield (71 %), mp 72-74 °C. \(^1\)H NMR (400 MHz, DMSO-d\(_6\)) \(\delta\) ppm 8.52 (d, J = 7.71 Hz, 1H), 7.68 (s, 1H), 7.57 (dd, J = 3.91, 7.91 Hz, 1H), 7.49 (d, J = 8.89 Hz, 1H), 7.34 - 7.47 (m, 2H), 7.31 (d, J = 6.54 Hz, 1H), 5.32 - 5.35 (m, 1H), 4.98 - 5.08 (m, 2H), 4.15 - 4.28 (m, 1H), 3.90 - 4.13 (m, 1H), 2.97 - 3.19 (m, 2H), 2.03 - 2.28 (m, 4H), 1.87 - 1.96 (m, 2H), 1.62 (d, J = 8.59 Hz, 7H), 1.39 - 1.52 (m, 4H), 1.21 - 1.35 (m, 6H), 1.05 - 1.15 (m, 4H), 0.81 - 0.93 (m, 2H). HRMS (ESI) calcd for C_{30}H_{43}ClN_{3}O_{9}S: [M-]: 656.2414. Found: 656.2403. HPLC purity 98.9% (Method A).
(5S,8S)-1-(3-Chlorophenyl)-5-(cyclohexylmethyl)-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-2,10-dioxa-4,7-diazaoctadecane-9-sulfonate (12): Yield (71%), mp 71-73 °C. ^1H NMR (400 MHz, DMSO-d$_6$) δ ppm 8.47 (d, J = 7.41 Hz, 1H), 7.53 - 7.62 (m, 1H), 7.49 (d, J = 8.49 Hz, 2H), 7.33 - 7.46 (m, 3H), 5.06 (br. s, 1H), 5.03 (s, 2H), 4.07 - 4.18 (m, 1H), 3.81 - 3.87 (m, 1H), 2.97 - 3.14 (m, 2H), 2.33 (br. s, 4H), 1.63 (d, J = 6.25 Hz, 6H), 1.38 - 1.47 (m, 4H), 1.19 - 1.31 (m, 10H), 1.10 (td, J = 7.10, 7.50 Hz, 6H), 0.86 (t, J = 6.54 Hz, 3H). HRMS (ESI) calcd for C$_{32}$H$_{47}$ClN$_3$O$_9$S: [M-]: 684.2727. Found: 684.2706. HPLC purity 97.3% (Method A).

(5S,8S)-1-(3-Chlorophenyl)-5-(cyclohexylmethyl)-12-methyl-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-2,10-dioxa-4,7-diazatridecane-9-sulfonate (13): Yield (41%), mp 76-78 °C. ^1H NMR (400 MHz, DMSO-d$_6$) δ ppm 8.50 (d, J = 7.71 Hz, 1H), 7.62 (s, 1H), 7.56 (d, J = 7.71 Hz, 1H), 7.34 - 7.45 (m, 3H), 7.30 (d, J = 4.59 Hz, 1H), 5.33 - 5.39 (m, 1H), 5.04 (d, J = 4.98 Hz, 2H), 4.15 - 4.23 (m, 1H), 4.10 (q, J = 7.94 Hz, 1H), 3.01 - 3.18 (m, 2H), 2.08 - 2.27 (m, 1H), 1.84 - 1.95 (m, 2H), 1.56 - 1.76 (m, 8H), 1.44 - 1.52 (m, 2H), 1.09 - 1.17 (m, 4H), 1.06 (d, J = 6.93 Hz, 6H), 0.82 - 0.93 (m, 2H). HRMS (ESI) calcd for C$_{28}$H$_{39}$ClN$_3$O$_9$S: [M-]: 628.2101. Found: 628.2115. HPLC purity 95.8% (Method A).

(5S,8S)-1-(3-Chlorophenyl)-11-cyclohexyl-5-(cyclohexylmethyl)-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-2,10-dioxa-4,7-diazaundecane-9-sulfonate (14): Yield (53%), mp 86-88 °C. ^1H NMR (400 MHz, DMSO-d$_6$) δ ppm 8.48 - 8.52 (m, 1H), 7.50 (d, J = 8.20 Hz, 1H), 7.44 (d, J = 3.91 Hz, 1H), 7.28 - 7.40 (m, 4H), 5.06 (s, 1H), 4.99 - 5.04 (m, 2H), 3.91 - 4.00 (m, 1H), 3.81 - 3.85 (m, 1H), 2.97 - 3.15 (m, 2H), 2.07 - 2.20 (m, 4H), 1.87 - 1.91 (m, 2H), 1.57 - 1.64 (m, 2H), 1.40 - 1.49 (m, 5H), 1.28 (s, 4H), 1.20 - 1.25 (m, 13H), 0.86 - 0.95 (m, 3H).
1.10 (td, \( J = 6.20, 6.50 \text{ Hz}, 4\text{H} \)), 0.80 - 0.91 (m, 8\text{H}). HRMS (ESI) calcd for C\(_{31}\)H\(_{43}\)ClN\(_3\)O\(_9\)S: [M\(-\text{H}\)]: 668.2144. Found: 668.2103. HPLC purity 97.7% (Method A).

**(S,S)-5-(Cyclohexylmethyl)-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-1-phenyl-2,10-dioxa-4,7-diazadodecane-9-sulfonate (15)**: Yield (62 \%), mp 92-94 °C.

\(^1\text{H NMR (400 MHz, DMSO-}d_6\text{)} \delta ppm 8.34 (d, \( J = 8.79 \text{ Hz}, 1\text{H}) \), 7.47 - 7.53 (m, 1\text{H}), 7.34 (br. s., 5\text{H}), 5.68 - 5.76 (m, 1\text{H}), 5.02 (s, 2\text{H}), 4.64 - 4.71 (m, 1\text{H}), 3.97 - 4.09 (m, 1\text{H}), 3.73 (q, \( J = 7.13 \text{ Hz}, 1\text{H}) \), 2.96 - 3.15 (m, 2\text{H}), 2.04 - 2.26 (m, 2\text{H}), 1.99 (s, 3\text{H}), 1.79 (d, \( J = 11.03 \text{ Hz}, 2\text{H}) \), 1.53 - 1.72 (m, 6\text{H}), 1.38 - 1.48 (m, 3\text{H}), 1.22 - 1.34 (m, 1\text{H}), 1.04 - 1.20 (m, 2\text{H}), 0.86 (d, \( J = 11.33 \text{ Hz}, 2\text{H}) \). HRMS (ESI) calcd for C\(_{26}\)H\(_{36}\)N\(_3\)O\(_9\)S: [M\(-\text{H}\)]: 566.2178. Found: 566.2147. HPLC purity 95.8% (Method A).

**(S,S)-11-Cyclohexyl-5-(cyclohexylmethyl)-3,6,11-trioxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-1-phenyl-2,10-dioxa-4,7-diazaundecane-9-sulfonate (16)**: Yield (52 \%), mp 83-85 °C.

\(^1\text{H NMR (400 MHz, DMSO-}d_6\text{)} \delta ppm 8.43 - 8.52 (m, 1\text{H}), 7.64 (d, \( J = 8.49 \text{ Hz}, 1\text{H}) \), 7.48 - 7.54 (m, 1\text{H}), 7.34 (br. s., 5\text{H}), 5.69 - 5.76 (m, 1\text{H}), 5.02 (s, 2\text{H}), 3.96 - 4.12 (m, 1\text{H}), 3.69 - 3.81 (m, 1\text{H}), 2.96 - 3.17 (m, 2\text{H}), 2.18 (t, \( J = 7.37 \text{ Hz}, 4\text{H}) \), 2.13 (br. s., 2\text{H}), 1.48 - 1.71 (m, 7\text{H}), 1.35 - 1.46 (m, 2\text{H}), 1.27 (d, \( J = 4.00 \text{ Hz}, 4\text{H}) \), 1.04 - 1.19 (m, 4\text{H}), 0.85 (qd, \( J = 5.20, 5.60 \text{ Hz}, 6\text{H}) \). HRMS (ESI) calcd for C\(_{26}\)H\(_{36}\)N\(_3\)O\(_9\)S: [M\(-\text{H}\)]: 634.2804. Found: 634.2818. HPLC purity 98.1% (Method A).

**Synthesis of compounds 17: General procedure.** Hexyl chloroformate (1.1 mmol) was added to a solution of dipeptidyl bisulfite salt (1 mmol) in dry acetonitrile (10 mL) and the reaction mixture was refluxed for 2 h with stirring. The solvent was removed in vacuo and the oily residue was treated with ether/hexane until a white precipitate
formed. The supernatant was carefully removed using a pipette and the white solid was
dried in vacuo.

(5S,8S)-1-(3-chlorophenyl)-5-(cyclohexylmethyl)-3,6,11-trioxo-8-((2-oxo pyrrolidin-3-yl)methyl)-2,10,12-trioxa-4,7-diazaoctadecane-9-sulfonate (17): Yield (61 %), mp 77-79 °C. \( ^{1} \text{H NMR (400 MHz, DMSO-d}_{6} \) \( \delta \) ppm 8.53 (d, \( J = 7.74 \) Hz, 1H), 7.68 (s, 1H), 7.57 (dd, \( J = 3.91, 7.91 \) Hz, 1H), 7.49 (d, \( J = 8.89 \) Hz, 1H), 7.34 - 7.47 (m, 2H), 7.31 (d, \( J = 6.54 \) Hz, 1H), 5.32 - 5.35 (m, 1H), 4.98 - 5.08 (m, 2H), 4.15 - 4.28 (m, 1H), 3.90 - 4.13 (m, 1H), 3.60 (t, \( J = 8.54 \) Hz, 2H), 2.97 - 3.19 (m, 2H), 2.03 - 2.28 (m, 4H), 1.87 - 1.96 (m, 2H), 1.62 (d, \( J = 8.59 \) Hz, 7H), 1.39 - 1.52 (m, 4H), 1.21 - 1.35 (m, 6H), 1.05 - 1.15 (m, 4H), 0.81 - 0.93 (m, 2H). HRMS (ESI) calcd for \( \text{C}_{31}\text{H}_{45}\text{ClN}_{3}\text{O}_{10}\text{S}: \) [M-]: 709.2412. Found: 709.2408. HPLC purity 98.9% (Method A).

Synthesis of compounds 18-21: General procedure. Isocyanate of amino acid (1.1 mmol) was added to a solution of dipeptidyl bisulfite salt (1 mmol) in dry acetonitrile (10 mL) and the reaction mixture was refluxed for 2 h with stirring. The solvent was removed in vacuo and the oily residue was treated with ether until a white precipitate formed. The supernatant was carefully removed using a pipette and the white solid was dried in vacuo.

(5S,8S)-5-Isobutyl-3,6,11,14-tetraoxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-1-phenyl-2,10,15-trioxa-4,7,12-triazahexadecane-9-sulfonate (18): Yield (67 %), mp 83-86 °C. \( ^{1} \text{H NMR (400 MHz, DMSO-d}_{6} \) \( \delta \) ppm 8.43 (d, \( J = 8.31 \) Hz, 1H), 7.59 (s, 1H), 7.40 - 7.52 (m, 1H), 6.48 (t, \( J = 6.91 \) Hz, 1H), 4.98 - 5.07 (m, 2H), 4.15 - 4.28 (m, 1H), 3.87 - 4.03 (m, 1H), 3.74 (s, 3H), 3.68 (d, \( J = 7.13 \) Hz, 1H), 3.35 (d, \( J = 7.03 \) Hz, 1H), 3.11 (dd, \( J = 8.84, 18.43 \) Hz, 2H), 2.02 - 2.26 (m, 2H), 1.53 - 1.74 (m, 2H), 1.37 -
1.54 (m, 3H), 1.05 - 1.16 (m, 2H), 0.86 (d, J = 7.33 Hz, 6H). HRMS (ESI) calcd for C_{25}H_{35}N_{4}O_{11}S: [M-]: 599.2029. Found: 599.2019. HPLC purity 96.3% (Method B).

(5S,8S)-5-Isobutyl-13-methyl-3,6,11,14-tetraoxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-1-phenyl-2,10,15-trioxa-4,7,12-triazahexadecane-9-sulfonate (19): Yield (70 %), mp 63-65 °C. ¹H NMR (400 MHz, DMSO-d₆) δ ppm 8.45 (d, J = 7.52 Hz, 1H), 7.60 (s, 1H), 7.46 (d, J = 7.81 Hz, 1H), 7.28 - 7.37 (m, 5H), 6.39 (d, J = 7.62 Hz, 1H), 5.01 (s, 2H), 4.34 - 4.41 (m, 1H), 4.13 - 4.25 (m, 1H), 4.06 (q, J = 7.20 Hz, 1H), 3.72 (s, 3H), 3.00 - 3.16 (m, 2H), 2.26 (dd, J = 3.66, 9.03 Hz, 1H), 1.87 (ddd, J = 4.05, 10.69, 14.35 Hz, 2H), 1.54 - 1.67 (m, 3H), 1.38 - 1.49 (m, 2H), 1.21 (d, J = 7.32 Hz, 3H), 1.07 (dt, J = 4.39, 7.08 Hz, 1H), 0.84 (d, J = 7.60 Hz, 6H). HRMS (ESI) calcd for C_{26}H_{37}N_{4}O_{11}S: [M-]: 613.2185. Found: 613.2174. HPLC purity 95.9% (Method B).

(5S,8S)-5-Isobutyl-13-isopropyl-3,6,11,14-tetraoxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-1-phenyl-2,10,15-trioxa-4,7,12-triazahexadecane-9-sulfonate (20): Yield (73 %), mp 85-87 °C. ¹H NMR (400 MHz, DMSO-d₆) δ ppm 8.48 (d, J = 7.52 Hz, 1H), 7.61 - 7.67 (m, 1H), 7.46 - 7.53 (m, 1H), 7.27 - 7.42 (m, 5H), 6.38 - 6.43 (m, 1H), 4.95 - 5.08 (m, 2H), 4.15 - 4.24 (m, 1H), 4.03 - 4.12 (m, 1H), 3.90 - 4.01 (m, 1H), 3.67 (s, 3H), 3.11 (dd, J = 6.35, 13.28 Hz, 2H), 2.31 - 2.35 (m, 1H), 2.07 - 2.15 (m, 3H), 1.96 - 2.05 (m, 2H), 1.91 (dd, J = 8.70, 14.20 Hz, 3H), 1.39 - 1.71 (m, 6H), 1.10 (dt, J = 4.49, 7.08 Hz, 1H), 0.76 - 0.92 (m, 6H). HRMS (ESI) calcd for C_{28}H_{41}N_{4}O_{11}S: [M-]: 641.2498. Found: 641.2464. HPLC purity 98.5% (Method B).

(5S,8S)-1-(3-Chlorophenyl)-5-(cyclohexylmethyl)-13-methyl-3,6,11,14-tetraoxo-8-(((S)-2-oxopyrrolidin-3-yl)methyl)-2,10,15-trioxa-4,7,12-triazahexadecane-9-sulfonate (21): Yield (66 %), mp 64-66 °C. ¹H NMR (400 MHz, DMSO-d₆) δ ppm 8.50
(d, J = 7.81 Hz, 1H), 7.61 - 7.69 (m, 1H), 7.48 - 7.57 (m, 1H), 7.34 - 7.44 (m, 2H), 7.30 
(d, J = 2.93 Hz, 1H), 6.42 (d, J = 7.42 Hz, 1H), 5.69 - 5.77 (m, 1H), 4.94 - 5.12 (m, 2H), 
4.19 - 4.29 (m, 1H), 4.06 - 4.17 (m, 1H), 3.81 - 4.00 (m, 1H), 3.63 (s, 3H), 2.96 - 3.20 (m, 
2H), 2.01 - 2.29 (m, 2H), 1.82 - 1.96 (m, 1H), 1.62 (d, J = 8.59 Hz, 6H), 1.38 - 1.52 (m, 
4H), 1.23 (d, J = 7.23 Hz, 3H), 1.06 - 1.14 (m, 4H), 0.76 - 0.94 (m, 2H). HRMS (ESI) 
calcd for C_{29}H_{40}ClN_{4}O_{11}S: [M-]: 687.2108. Found: 687.2131. HPLC purity 97.4% 
(Method B).

(5S,8S)-1-(3-Chlorophenyl)-5-(cyclohexylmethyl)-13-isopropyl-3,6,11,14-tetraoxo- 
8-(((S)-2-oxopyrrolidin-3-yl)methyl)-2,10,15-trioxa-4,7,12-triazahexadecane-9- 
sulfonate (22): Yield (62 %), mp 82-84 °C. ^1H NMR (400 MHz, DMSO-d_6) δ ppm 8.45 
(d, J = 7.72 Hz, 1H), 7.63 - 7.71 (m, 1H), 7.48 - 7.55 (m, 1H), 7.31 - 7.42 (m, 2H), 7.20 
(d, J = 3.56 Hz, 1H), 6.47 (d, J = 7.61 Hz, 1H), 5.62 - 5.74 (m, 1H), 4.94 - 5.12 (s, 2H), 
4.19 - 4.31 (m, 1H), 4.06 - 4.17 (m, 1H), 3.81 - 4.02 (m, 1H), 3.63 (s, 3H), 2.96 - 3.17 (m, 
2H), 2.01 - 2.29 (m, 2H), 1.82 - 1.96 (m, 1H), 1.62 (m, 6H), 1.38 - 1.52 (m, 4H), 1.10 (dt, 
J = 4.49, 7.08 Hz, 1H), 1.06 - 1.14 (m, 4H), 0.97-1.04 (d, J= 7.48 Hz, 6H), 0.76 - 0.94 (m, 
2H). HRMS (ESI) calcd for C_{31}H_{44}ClN_{4}O_{11}S: [M-]: 715.2421. Found: 715.2413. HPLC 
purity 98.3% (Method B).

(5S,8S)-5-(Cyclohexylmethyl)-13-methyl-3,6,11,14-tetraoxo-8-(((S)-2-oxopyrrolidin- 
3-yl)methyl)-1-phenyl-2,10,15-trioxa-4,7,12-triazahexadecane-9-sulfonate (23) : Yield (72 %), mp 61-63 °C. ^1H NMR (400 MHz, DMSO-d_6) δ ppm 8.47 (s, 1H), 7.63 (s, 
1H), 7.42 - 7.54 (m, 1H), 7.37 (s, 1H), 7.28 - 7.40 (m, 5H), 6.52 (t, J = 5.91 Hz, 1H), 4.98 
- 5.04 (m, 2H), 4.15 - 4.28 (m, 1H), 3.87 - 4.03 (m, 1H), 3.77 (s, 3H), 3.74 (d, J = 7.13 
Hz, 1H), 3.11 (dd, J = 8.84, 19.67 Hz, 2H), 2.02 - 2.22 (m, 2H), 1.53 - 1.70 (m, 4H), 1.34
- 1.52 (m, 4H), 1.05 - 1.13 (d, J = 7.24 Hz, 3H), 0.87 - 0.98 (m, 6H), 0.76 - 0.84 (m, 2H).

HRMS (ESI) calcd for C_{29}H_{41}N_{4}O_{11}S: [M-]: 653.2498. Found: 653.2483. HPLC purity 97.7% (Method B).

**Enzyme assays and inhibition studies. FRET protease assays.** The FRET protease assay (3CLpro) was performed by preparing stock solutions of the substrate (Edans-DFHLQ/GP-Dabcyl) and inhibitor in DMSO and diluting into assay buffer which was comprised of 20 mM HEPES buffer, pH 8, containing NaCl (200 mM), 0.4 mM EDTA, glycerol (60%), and 6 mM dithiothreitol (DTT). The protease was mixed with serial dilutions of each compound up to 100 µM or with DMSO in 25 µL of assay buffer and incubated at 37°C for 30 min, followed by the addition of 25 µL of assay buffer containing substrate. Fluorescence readings were obtained using an excitation wavelength of 360 nm and an emission wavelength of 460 nm on a fluorescence microplate reader (FLx800; Biotec, Winoosk, VT) 1 h following the addition of substrate. Relative fluorescence units (RFU) were determined by subtracting background values (substrate-containing well without protease) from the raw fluorescence values, as described previously. The dose-dependent FRET inhibition curves were fitted with a variable slope by using GraphPad Prism software (GraphPad, La Jolla, CA) in order to determine the IC_{50} values of the inhibitors.

**Cell-based inhibition assays.** The effects of each inhibitor on virus replication were examined against NV in the NV replicon harboring cells (HG23 cells). Briefly, confluent and semi-confluent HG23 cells were incubated with medium containing DMSO (<0.1%) or each compound (up to 20 µM) for 48 h. After the incubation, total RNA was extracted
and viral genome was quantitated with real-time quantitative RT-PCR (qRT-PCR). The 
EC$_{50}$ values were determined by GraphPadPrism software.$^{26}$

**Cytotoxicity.** The cytotoxicity of a select number of compounds was determined using 
human HeLa cells and the MTT assay as described previously.$^{52}$ This assay measures 
the viability of a population of cells after a 72 h incubation with the test compound. The 
CC$_{50}$ was calculated as the concentration at which cell viability was at 50% of the 
untreated controls.

**Liver microsomes stability.** Liver microsomes stability was determined by incubating 
25 µM compound in a solution with mouse or human liver microsomes (Xenotech), 
NADPH (Sigma Aldrich) 50 mM phosphate buffer (pH 7.4) and 3 mM MgCl$_2$ for 1 h at 
37°C, as described previously,$^{53}$ quenching the sample with 1% trifluoroacetic acid in 
acetonitrile and then analyzing the quantity of the parent compound present in the 
sample using LC/MS (Thermo Scientific LCQ Fleet).

**Plasma Protein-Binding.** Protein binding studies were performed using a 96-well 
equilibrium dialysis apparatus (Pierce Biochemicals) according to a published 
protocol.$^{54}$ Mouse or human serum samples (55%) were incubated at 37 °C for 24 h in 
the presence of 25 µM compound prior to LC/MS quantitation of the post-dialysis 
material.

**Formation of aldehyde 25 in the cell-based assay.** HG23 cells were treated with 
compound 11 (50 µM) for 24 h. All cells and medium containing inhibitor 11 were 
sonicated, followed by centrifugation for 5 minutes. The supernatant was removed and 
an aliquot (4 µL) was diluted in a mixture of water and 0.1% formic acid (200 µL) and
desalted with a µ-C18 Zip Tip prior to infusing (0.3 µL/min) into Thermo LTQ Orbitrap XL mass spectrometer.

**High Performance Liquid Chromatography (HPLC).**

**Method A:** Prodrug compound 1 mg was dissolved in 100 µL of acetonitrile: dichloromethane (2:3) mixture. Then 10 µL of solution (10 g/L) was injected into HPLC (Varian Pro-star) with mobile phase isocratic 40 mode with 40% acetonitrile and 60% dichloromethane. The flow rate of mobile phase is 1.0 mL/min through the normal phase column stationary phase (Kinetex 2.6u HILIC 100A, 75 x 4.6mm).

**Method B:** Prodrug compound 1 mg was dissolved in 100 µL of acetonitrile: dichloromethane (2:3) mixture. Then 10 µL of solution (10 g/L) was injected into HPLC (Varian Pro-star) with mobile phase isocratic 20 mode with 20% acetonitrile and 80% dichloromethane. The flow rate of mobile phase is 1.0 mL/min through the normal phase column stationary phase (Kinetex 2.6u HILIC 100A, 75 x 4.6mm).

**ASSOCIATED CONTENTS**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: Molecular Formula Strings-SMILES Codes (CSV).

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Notes

The authors declare no competing financial interests.

ACKNOWLEDGMENTS

The generous financial support of this work by the National Institutes of Health (AI109039) is gratefully acknowledged. We thank Mr. Matthew A. Baird for assistance with the mass spectrometric analyses.

ABBREVIATIONS USED

PK, pharmacokinetics; DMF, N,N-dimethyl formamide; TLC, thin layer chromatography; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, N-hydroxybenzotriazole; DIEA, diisopropylethylamine; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; MNV, murine norovirus; TCID<sub>50</sub>, 50% tissue culture infectious dose; IC<sub>50</sub>, 50% inhibitory concentration in the enzyme assay; EC<sub>50</sub>, 50% effective concentration in cell culture; CC<sub>50</sub>, 50% cytotoxic concentration in cell-based assays; TS, transition state.
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(27) Kim, Y.; Lovell, S.; Tiew, K-C.; Mandadapu, S. R.; Alliston, K. R.; Battaile, K. P.; Groutas, W. C.; Chang, K-O. Broad-spectrum antivirals against 3C or 3C-like


(51) The nomenclature used is that of Schechter, I. & Berger, A. On the size of the active site in proteases. I. Papain. Biochem. Biophys. Res. Comm. 1967, 27, 157-162 where the residues on the N-terminus side of the peptide bond that is cleaved are designated as P₁-Pₙ and those on the C-terminus side are designated P₁‘-Pₙ‘. The corresponding active site subsites are designated S₁-Sₙ and S₁‘-Sₙ‘. S₁ is the primary substrate specificity subsite and P₁-P₁‘ is the scissile bond.


Table 1. Anti-NV Activity of Compounds 1-17 in Enzyme (IC$_{50}$) and Cell-Based (EC$_{50}$) Assay.
<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>IC₅₀ (µM)</th>
<th>EC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td></td>
<td>CH₃</td>
<td>10.5</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>CH₂CH₃</td>
<td>7.5</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>(CH₂)₂CH₃</td>
<td>3.7</td>
<td>1.3</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>(CH₂)₃CH₃</td>
<td>3.5</td>
<td>1.4</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>(CH₂)₄CH₃</td>
<td>2.8</td>
<td>0.3</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>(CH₂)₆CH₃</td>
<td>3.0</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>C₆H₅</td>
<td>7.2</td>
<td>0.8</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>CH(CH₃)₂</td>
<td>3.5</td>
<td>0.3</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>m-Cl</td>
<td>CH₃</td>
<td>0.08</td>
<td>0.03</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>CH₂CH₃</td>
<td>0.1</td>
<td>0.025</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td>(CH₂)₄CH₃</td>
<td>0.1</td>
<td>0.015</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td>(CH₂)₆CH₃</td>
<td>0.9</td>
<td>0.03</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td>CH(CH₃)₂</td>
<td>0.3</td>
<td>0.03</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td>C₆H₁₁</td>
<td>0.4</td>
<td>0.02</td>
</tr>
<tr>
<td>15</td>
<td>H</td>
<td></td>
<td>CH₃</td>
<td>7.5</td>
<td>0.2</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>m-Cl</td>
<td>(CH₂)₄CH₃</td>
<td>0.6</td>
<td>0.06</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>m-Cl</td>
<td>O(CH₂)₆CH₃</td>
<td>0.8</td>
<td>0.025</td>
</tr>
</tbody>
</table>

**Table 2.** Anti-NV Activity of Compounds 18-23 in Enzyme (IC₅₀) and Cell-Based (EC₅₀)
Table 3. Hydrolysis of Compounds 9-11, 24 and 26 in Mouse or Human Serum to Yield the Precursor Aldehyde.
<table>
<thead>
<tr>
<th>Compound</th>
<th>% of parent remaining after 1 h</th>
<th>Mouse Serum</th>
<th>Human Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60</td>
<td>103</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Compounds (25 µM or 100 µM) were incubated with 55% serum for 1 h at 37°C (detection of aldehyde by LC/MS).

<sup>b</sup>Structures shown in Figure 4.

<sup>c</sup>See Table 1 for structures.
**Table 4.** Cytotoxicity and Selectivity Index of Compounds 9-11 and 24-26.

<table>
<thead>
<tr>
<th>Compound&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;200</td>
<td>0.3</td>
<td>&gt;667</td>
</tr>
<tr>
<td>25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.1</td>
<td>0.02</td>
<td>2,800</td>
</tr>
<tr>
<td>26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49.6</td>
<td>0.02</td>
<td>2,480</td>
</tr>
<tr>
<td>9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&gt;200</td>
<td>0.03</td>
<td>&gt;6,667</td>
</tr>
<tr>
<td>10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>112</td>
<td>0.025</td>
<td>4480</td>
</tr>
<tr>
<td>11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>82</td>
<td>0.015</td>
<td>5,467</td>
</tr>
</tbody>
</table>

<sup>a</sup>HeLa cells were exposed to compound during a 72-h incubation and cytotoxicity was determined using the MTT assay.

<sup>b</sup>Structures shown in Figure 4

<sup>c</sup>See Table 1 for structures
### Table 5. Metabolic Stability of Compounds 9-11 and 25-27 with Human and Mouse Liver Microsomes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% of parent remaining after 1 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse Microsomes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82</td>
</tr>
<tr>
<td>25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40</td>
</tr>
<tr>
<td>26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35</td>
</tr>
<tr>
<td>9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14</td>
</tr>
<tr>
<td>10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17</td>
</tr>
<tr>
<td>11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38</td>
</tr>
<tr>
<td>Warfarin (high stability control)</td>
<td>99.6</td>
</tr>
<tr>
<td>Verapamil (low stability control)</td>
<td>30</td>
</tr>
</tbody>
</table>

<sup>a</sup>Compounds (25 µM or 100 µM) were incubated with mouse or human liver microsomes in the presence of NADPH for 1 h at 37°C (detection of aldehyde by LC/MS).

<sup>b</sup>Structures listed in Figure 4.

<sup>c</sup>See Table 1 for structures.
**Table 6.** Plasma Protein Binding of Compounds 9-11 and 24-26.

<table>
<thead>
<tr>
<th></th>
<th>% bound to serum protein</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse Serum(^a)</td>
<td>Human Serum(^a)</td>
<td></td>
</tr>
<tr>
<td>24(^b)</td>
<td>71</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>25(^b)</td>
<td>98</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>26(^b)</td>
<td>97</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>9(^c)</td>
<td>ND(^d)</td>
<td>ND(^d)</td>
<td></td>
</tr>
<tr>
<td>10(^c)</td>
<td>ND(^d)</td>
<td>ND(^d)</td>
<td></td>
</tr>
<tr>
<td>11(^c)</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Atenolol (weak binding control)</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Warfarin (strong binding control)</td>
<td>96</td>
<td>99</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Compound (25 µM or 100 µM) was incubated with 55% mouse or human serum in an equilibrium dialysis chamber for 4 h at 37°C (detection of aldehyde by LC/MS).

\(^b\)Structures shown in Figure 4.

\(^c\)See Table 1 for structures.

\(^d\)ND, not detected due to low serum stability over 4 h.
Scheme 1

\[ \text{Scheme 1} \]

- **Scheme 1**

  a, b

  \[
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{OH} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H}
  \]

- **Scheme 1**

  c

  \[
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{OH} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H}
  \]

- **Scheme 1**

  d

  \[
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{OH} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H}
  \]

- **Scheme 1**

  e

  \[
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{OH} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H}
  \]

- **Scheme 1**

  f

  \[
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{OH} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H} \\
  \text{R} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{C} \quad \text{H}
  \]

- **Scheme 1**

  a. EDCI/HOBt/DIEA/DMF then Gln surrogate; b. 2M LiBH4/THF/CH3OH; c. Dess-Martin periodinane/DCM; d. C2H5OH/EtOAc/NaHSO4; e. Alkanoic anhydride/CH3CN/Reflux 2 h; f. Amino acid isocyanate/CH3CN/Reflux 2 h.
Scheme 2

- **a,b** Trichloromethyl chloroformate/1,4-dioxane/Reflux 16h
- **c,d** 1M LiOH/THF/RT 3h
- **e** Dess-Martin periodinane/DCM
- **f** Chloroformate/CH$_3$CN/Reflux 2h
- **g** Amino acid isocyanate/CH$_3$CN/Reflux 2h
- **h** EDCI/HOBt/DIEA/DMF then Gln surrogate; 2M LiBH$_4$/THF/CH$_3$OH
- **i** Na$_2$S$_2$O$_3$/Alkanoic anhydride/CH$_3$CN/Reflux 2 h

\begin{align*}
(R_e=Cl) & \quad 9-14 \\
(R_e=H) & \quad 15-16 \\
(R_e=Cl) & \quad 17 \\
(R_e=Cl) & \quad 21-22 \\
\end{align*}
Figure 1

(I)  

(II)
Figure 2

\[
\begin{align*}
\text{O} & \quad \text{R} \\
X & \quad \text{Z} \\
\text{chemical/enzymatic cleavage} & \quad \rightarrow \\
\text{O} & \quad \text{Z} \\
\text{E-Cys-SH} & \quad \rightarrow \\
\text{R} & \quad \text{alkyl or alkoxy} \\
(\text{Z} = \text{H}, (\text{C}=\text{O})\text{NHR, COOR})
\end{align*}
\]
Figure 3
Figure 4

![Graph showing the remaining percentage of compounds over time. The graph plots time (in minutes) on the x-axis and the remaining percentage on the y-axis. The graph includes three lines, each representing a different compound: Compound 9, Compound 10, and Compound 11.]
Figure 5

![Chemical Structures](image)

**24**
- IC$_{50}$ = 0.8 µM
- EC$_{50}$ = 0.3 µM

**25**
- IC$_{50}$ = 0.1 µM
- EC$_{50}$ = 0.02 µM

**26**
- IC$_{50}$ = 0.1 µM
- EC$_{50}$ = 0.02 µM
LEGENDS TO FIGURES

Figure 1. General structures of prodrugs (I-II)

Figure 2. Postulated mechanism of action of prodrugs (I-II).

Figure 3. Panel A: hydrogen bond interactions of norovirus 3CL protease and precursor aldehyde derived from compound 25. Hydrogen bonds are represented as dashed lines and water mediated contacts are shown as solid lines. The catalytic Cys139 residue is shown covalently bound to the aldehyde warhead carbon. Panel B: surface representation of norovirus 3CL protease with inhibitor bound to the active site. Neighboring residues of norovirus 3CL protease are colored yellow (nonpolar), white (weakly polar) and cyan (polar) (reproduced from ref 26).

Figure 4. Time-dependent hydrolysis of prodrugs 9, 10 and 11 and conversion to the aldehyde form in 55% human serum.

Figure 5. Structures and potencies of compounds 24-26.
Graphical Abstract
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