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Potent inhibition of norovirus by dipeptidyl α-hydroxyphosphonate transition state mimics

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ABSTRACT

The design, synthesis, and evaluation of a series of dipeptidyl α-hydroxyphosphonates is reported. The synthesized compounds displayed high anti-norovirus activity in a cell-based replicon system, as well as high enzyme selectivity.

Noroviruses are the most common cause of acute viral gastroenteritis in the US, and worldwide,[1–3] accounting for ~21 million cases of gastroenteritis annually in the US alone.[4] Noroviruses are very stable in the environment and refractory to many common disinfectants, with only a few virions required to initiate virus infection. Therefore, norovirus outbreaks are hard to contain using routine sanitation, and even implementation of aggressive sanitary measures often fails to prevent subsequent norovirus outbreaks. The problem is further compounded by the lack of norovirus-specific antiviral agents or effective vaccines.[5] The challenges associated with vaccine development, such as high viral diversity and short-term immunity,[6] and the need for aggressive sanitary measures for combating continuous outbreaks of norovirus-associated gastroenteritis, render norovirus infection a serious public health problem and underscores the importance of developing small molecule anti-norovirus therapeutics and prophylactics.

Noroviruses have a single-stranded, positive sense 7–8 kb RNA genome that encodes a polyprotein precursor that is processed by a virus-encoded 3C-like cysteine protease (3CLpro) to generate mature non-structural proteins. Processing of the polyprotein by 3CLpro is essential for virus replication.[7] We have recently described the structure-based design, synthesis, and evaluation of transition state inhibitors of norovirus 3CLpro, including peptidyl aldehydes,[8] peptidyl α-ketoamides and α-ketoheterocycles and their corresponding bisulfite salts,[9,10] and macrocyclic inhibitors.[11] These studies have demonstrated that representative members of these classes of compounds potently inhibit norovirus 3CL proteases from various genogroups and exhibit significant anti-norovirus activity in a cell-based replicon system.[12] Taken together, the results of these studies are strongly supportive of the notion that norovirus 3CLpro is a druggable target that is well-suited to the discovery and development of anti-norovirus small molecule therapeutics and prophylactics.

In continuing our endeavors in this area,[8–13] we describe herein the results of preliminary studies related to the inhibition of 3CLpro by peptidyl α-hydroxyphosphonates (Fig. 1, structure (I)). To our knowledge, this is the first time that α-hydroxyphosphonate transition state mimics have been used in the inhibition of a viral cysteine protease.

The design of inhibitor **(I)** rested on the following considerations: (a) previous studies have shown that the α-hydroxyester[14] and α-hydroxyphosphonate[15] moieties function as effective transition state mimics which yield highly potent inhibitors when linked to a peptidyl recognition element that is tailored to the substrate specificity of a target protease. This approach has been successfully used in the design of highly effective inhibitors of human renin;[15] (b) NV 3CLpro is a cysteine endoprotease with a chymotrypsin-like fold, a His-Cys-Glu triad, and an extended binding site.[12,16–18] Mapping of the active site of 3CLpro using chromogenic and fluorogenic

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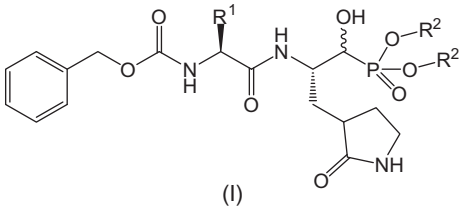


(I)

Figure 1. General structure of inhibitor (**I**).

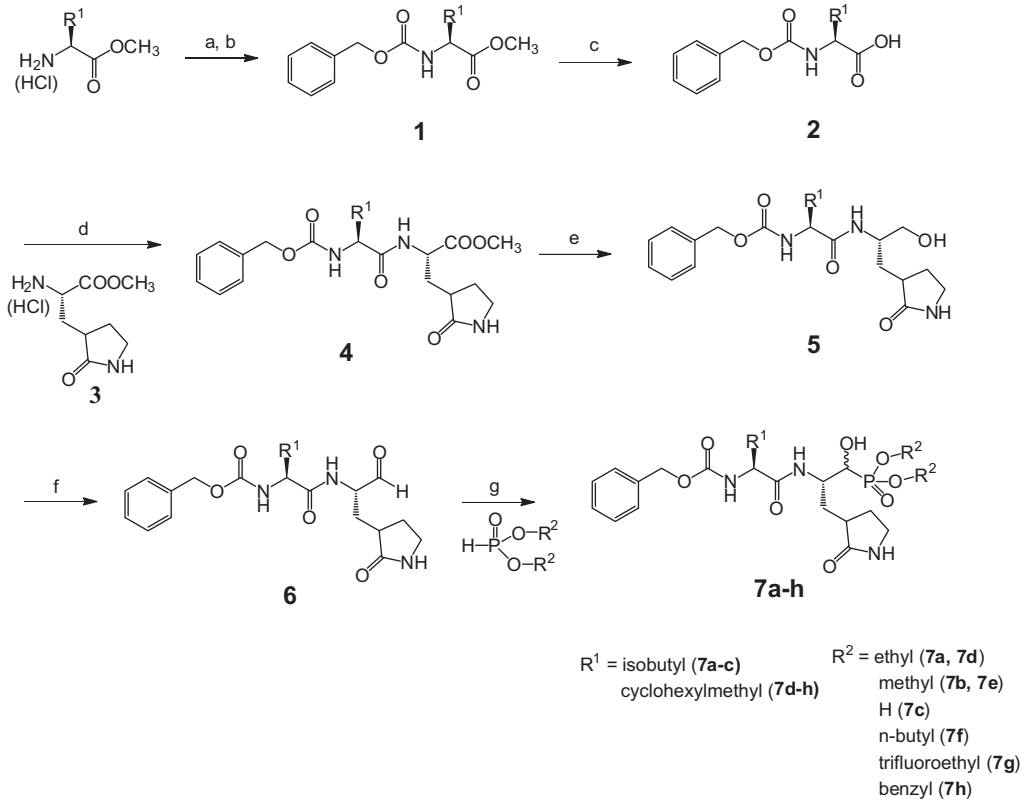
substrates has shown that the protease has a strong preference for a -D/E-F/Y-X-L-Q-G- sequence, where X is H, E or Q, and cleavage is at the Q-G (P_1-P_1')[19] bond;[16,20–22] (c) we have previously demonstrated that the presence of a P_2 cyclohexyl alanine residue results in a significant enhancement in potency and cellular permeability.[10,23] Based on the aforementioned considerations, it was envisaged that an inhibitor represented by general structure **(I)** (Fig. 1) may display high anti-norovirus activity in a cell-based replicon system mediated via the inhibition of NV 3CLpro.

Inhibitor **(I)** was readily constructed by transforming commercially available (L) cyclohexyl alanine to the corresponding ester and subsequent conversion of the ester to the isocyanate by refluxing with trichloromethyl chloroformate in dioxane (Scheme 1).

Subsequent reaction with an appropriate alcohol yielded the N-protected amino acid ester which was hydrolyzed with LiOH in aqueous THF to yield the corresponding acid, which was then coupled to the methyl ester of a previously-reported P_1 glutamine surrogate.[24] Reduction of the N-protected amino acid ester with lithium borohydride, followed by oxidation with Dess–Martin periodinane reagent,[25] yielded the corresponding dipeptidyl aldehyde which was reacted with an array of dialkyl phosphites and diisopropylethylamine in dichloromethane to yield the desired compounds as mixtures of epimers[26,27] (listed in Table 1). The reaction sequence chosen is highly tractable and permits facile modification of the cap by reaction with an array of structurally-diverse alcohols. This is of paramount importance in terms of optimizing pharmacological activity, ADMET, and PK via cap modifications.[28] The inhibitory activity of the generated α-hydroxyphosphonate derivatives was evaluated against norovirus in a cell-based replicon system.[29–32] The selectivity of a representative member of this series of compounds was also assessed using a panel of proteases (Table 2).

It is clearly evident from the results summarized in Table 1 that α-hydroxyphosphonates exhibit potent anti-norovirus activity in a cell-based replicon system. Consistent with previous findings,[10] the replacement of the P2 leucine with cyclohexyl alanine enhanced the inhibitory activity of the compounds (compare **7a**/**7d** and **7b**/**7e** in Table 1). The effect of varying R^2 (assumed to be projecting toward the S' subsites) on potency was also explored. With the exception of compound **7e**, variations in R^2 yielded compounds with submicromolar EC_{50} values, with compounds **7d** and **7g** having the highest potency.

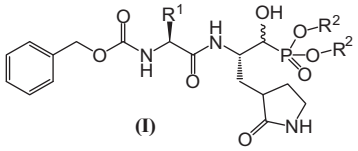
Toxicity arising from off-target effects is a potential caveat in viral protease drug development since the inhibitors may also inhibit host proteases. Thus, the selectivity profile of α-hydroxyphosphonate (**7d**) was investigated using a representative panel of proteases (Table 2). Compound **7d** was devoid of any inhibitory



Reagents: (a) $CCl_3O(C=O)Cl$/dioxane; (b) Triethylamine/R^1OH; (c) LiOH/THF/H_2O; (d) EDCI/HOBt/DIEA/DMF; (e) $LiBH_4$/THF; (f) Dess-Martin periodinane/DCM; (g) DIEA/DCM

Scheme 1. Synthesis of compounds **7a**–**h**.

Table 1
Anti-norovirus activity of inhibitor (I)



(I)

Compound	R^1	R^2	IC_{50} μM[a]	EC_{50} μM[a]
7a	Isobutyl	Ethyl	11.5	0.8
7b	Isobutyl	Methyl	20.2	3.5
7c	Isobutyl	H	ND[b]	7.5
7d	Cyclohexylmethyl	Ethyl	3.5	0.25
7e	Cyclohexylmethyl	Methyl	8.3	2.8
7f	Cyclohexylmethyl	*n*-Butyl	ND[b]	0.5
7g	Cyclohexylmethyl	Trifluoroethyl	6.5	0.35
7h	Cyclohexylmethyl	Benzyl	ND[b]	0.6

[a] Determined as described in Ref. 12.
[b] ND: not determined.

Table 2
Enzyme selectivity of compound **7d**

Enzyme	Inhibition[a] (%)
HNE[b,36]	15 ($[I_f]$ = 17.5 μM)[c]
α-Chymotrypsin[37]	0 ($[I_f]$ = 2.5 μM)
Trypsin[38]	0 ($[I_f]$ = 125 μM)
Carboxypeptidase A[39]	0 ($[I_f]$ = 43 μM)
Thrombin[40]	0 ($[I_f]$ = 2.75 μM)

[a] Determined by incubating enzyme and inhibitor in buffer solution for 30 min at an [I]/[E] ratio of 250.
[b] HNE, human neutrophil elastase.
[c] Final inhibitor concentration.

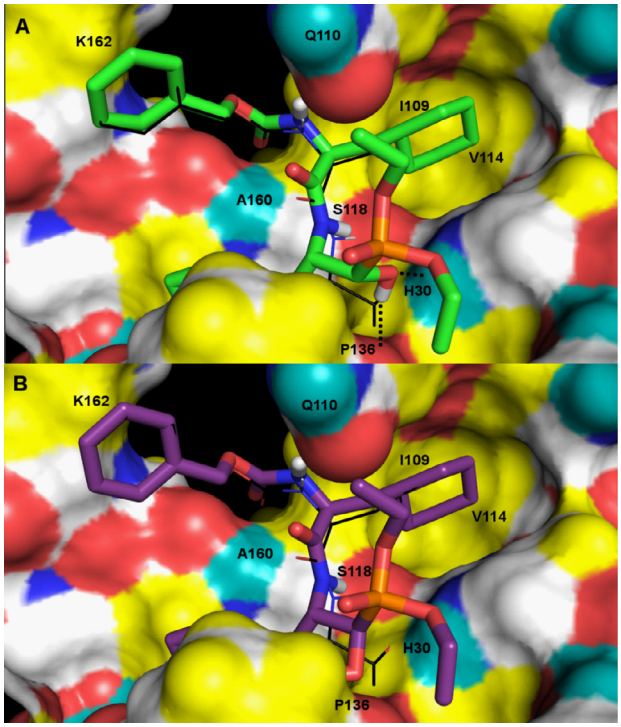


Figure 2. Computationally predicted conformers for noncovalent tetrahedral mimics A (α-OH) (CPK colored sticks, with green carbons) and B (β-OH) (CPK sticks; purple carbons) bound to the catalytic site of NV 3C protease (Connolly surface colored as follows: yellow = nonpolar groups; white = weakly polar alkyl and aryl groups; cyan = polar H's, blue = polar N's and red = polar O's). In both cases, the predicted conformer of the corresponding tetrahedral adduct (thin sticks; CPK colors with black carbons is shown for reference.

activity against basic serine (trypsin, thrombin) and metallo- (carboxypeptidase A) proteases, as well as no or very low activity against neutral serine proteases (α-chymotrypsin, human neutrophil elastase), attesting to the high enzyme selectivity of this class of compounds.

In order to gain insight and understanding into the binding of epimers **A** (α-OH)) and **B** (β-OH), molecular mechanics simulations using the Avogadro program[33] (MMFF94 potentials and electrostatics[34] were used to qualitatively assess the relative affinities of compounds **A** and **B** (Fig. 2). To accomplish this, the receptor model was crafted using a recent crystal structure of NV 3C protease with a bound peptidic ligand[16] by removing water molecules and adding protons (per physiological pH) using the PyMol program.[35] A preliminary model for the bound conformation of the tetrahedral adduct was built within the receptor using Avogadro, as a covalent extension to Cys 139 that mimicked the conformation of the cocrystallized inhibitor from the 2IPH structure and placed the ligand cyclohexyl group in the hydrophobic pocket occupied by the leucyl side chain of the peptidyl inhibitor. The resulting model was subjected to 500 steps of molecular mechanics energy minimization. Structures of **A** and **B** were then constructed from the adduct in Avogadro by deleting the covalent attachment to Cys 139, specifying the hydroxyl and the diethyl phosphonate groups in a manner commensurate with the stereochemistry of **A** and **B** and re-optimizing the resulting structures (again for 500 steps). The results suggest that inhibitory activity resides primarily with epimer **A** which is capable of additional binding interactions with the enzyme (Fig. 2).

In summary, we report herein for the first time the cell-based anti-norovirus activity and enzyme selectivity of a series of dipeptidyl α-hydroxyphosphonates.

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