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STRUCTURE-BASED DESIGN OF IRREVERSIBLE, TRIPEPTIDYL HUMAN RHINOVIRUS 3C PROTEASE INHIBITORS CONTAINING *N*-METHYL AMINO ACIDS

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Abstract. Tripeptide-derived molecules incorporating *N*-methyl amino acid residues and C-terminal Michael acceptor moieties were evaluated as irreversible inhibitors of the cysteine-containing human rhinovirus 3C protease (3CP). Such compounds displayed good 3CP inhibition activity (k_{obs}/[I] up to 610,000 $M^{-1}s^{-1}$) and potent *in vitro* antiviral properties (EC_{50} approaching 0.03 μM) when tested against HRV serotype-14.

The human rhinoviruses (HRVs) are members of the picornavirus family and are the single most significant cause of the common cold.[1,2] The replication of these viruses is entirely dependent on the proteolytic processing of a large polyprotein produced by cellular translation of the viral RNA genome. This processing is primarily accomplished by the human rhinovirus 3C protease (3CP),[3] a cysteine protease with structural similarity to the trypsin protein family but possessing minimal homology to prevalent mammalian enzymes.[4] Due to its prominence in the viral replication cycle, 3CP is an ideal target for the development of novel antirhinoviral agents. Indeed, several examples of 3CP inhibitors have appeared in the literature, including peptide aldehydes,[5] isatins,[6] homophthalimides,[7] and other miscellaneous entities.[8] Recently, we described the discovery and development of a new class of 3CP inhibitors which display *in vitro* antiviral activity against several rhinovirus serotypes.[9,10] These inhibitors are comprised of a substrate-derived[3] tripeptide binding determinant which provides affinity for the target protease and a Michael acceptor moiety which irreversibly forms a covalent adduct with the active site cysteine residue of the 3C enzyme (e.g., compound **1**).[11,12] In this report, we describe the further exploration of such irreversible 3CP inhibitors by the incorporation of *N*-methyl amino acids into the inhibitor design.

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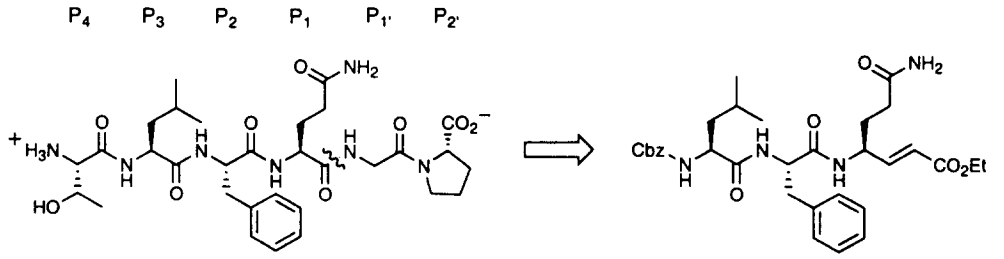
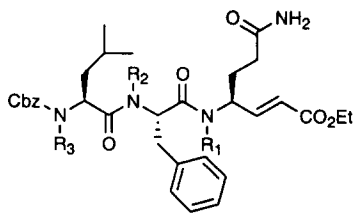


As a routine exercise during peptide structure-activity studies, we introduced methyl groups along the amide backbone of a typical tripeptidyl 3CP inhibitor (compound **1**). This study was performed even though analysis of the HRV-2 3CP-**1** X-ray crystal structure[9] indicated that all inhibitor backbone amide NHs formed hydrogen bonds with the enzyme. As expected from the X-ray analysis, inclusion of an *N*-methyl amino acid at the P_1 or P_3 position[13] within the inhibitor design resulted in drastic reduction in anti-3CP activity[14] relative to the non-methylated compound (compare **2** and **4** with **1**, Table 1). However, a molecule containing a P_2 (*N*-Me)Phe[15] residue surprisingly displayed only moderately reduced 3CP inhibitory activity and was active in a cell-based antirhinoviral assay (compound **3**, Table 1).[16,17] This result paralleled the previous observation that replacement of the same P_2-P_3 amide linkage with a ketomethylene isostere[18] resulted in minimal loss of anti-3CP activity, although it was not anticipated that the bulky methyl group could be so readily accommodated in the enzyme active site.[19] In any event, the anti-3CP and antiviral activity displayed by compound **3** prompted an extensive investigation of inhibitors containing P_2 *N*-methyl amino acids.

Table 1.



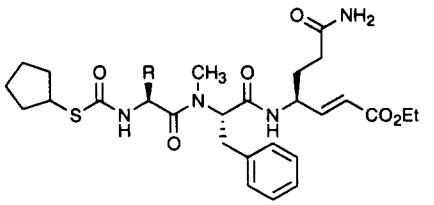
Compd.	R_1	R_2	R_3	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)[a]	EC_{50} (μM)[a]
1	H	H	H	25,000	0.54
2	CH_3	H	H	NI	ND
3	H	CH_3	H	5,300	1.0
4	H	H	CH_3	100	ND

[a]Serotype-14. NI = no inhibition to 50 μM; ND = not determined.

Accordingly, modifications previously shown to improve the anti-3CP and/or antirhinoviral activity of non-methylated tripeptidyl 3CP inhibitors were then incorporated into the P_2 *N*-methylamide-containing series. Thus, substitution of an N-terminal *S*-cyclopentyl thiocarbamate for the Cbz moiety of **3** improved both 3CP inhibition and antiviral properties in direct analogy with earlier structure-activity studies (compound **5**, Table 2).[10] However, in contrast to results observed with non-methylated tripeptides, inclusion of a Val residue at the P_3 position within the inhibitor design did not significantly improve anti-3CP activity relative to the Leu-containing compound (compare **5** with **6**, Table 2).[20] This activity difference probably results from a steric clash between the Val side chain and the P_2 *N*-methyl moiety which attenuates the beneficial effects of P_3 Val incorporation. A

P_3 *S*-phenyl cysteine residue was also combined with an N-terminal thiocarbamate moiety, and the resulting compound (**7**) displayed good levels of both anti-3CP and antirhinoviral activity.

Table 2.

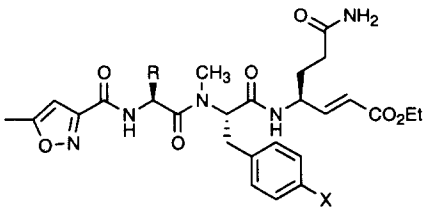


Compd.	R	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)[a]	EC_{50} (μM)[a]
5	$CH_2CH(CH_3)_2$	17,320	0.32
6	$CH(CH_3)_2$	22,500	0.19
7	CH_2SPh	36,800	0.54

[a]Serotype-14.

In addition to the molecules described above, we examined several P_2 *N*-methylamide-containing tripeptides which incorporated an N-terminal amide derived from 5-methylisoxazole-3-carboxylic acid (Table 3).[21] Substitution of the Cbz moiety of **3** with such an amide improved both 3CP inhibition activity and antiviral properties in analogy with previous SAR studies (compound **8**). Surprisingly, incorporation of a P_2 (4-F,*N*-Me)Phe residue into the inhibitor design resulted in somewhat diminished anti-3CP activity (compound **9**). This result was again in contrast with that observed for non-methylated tripeptidyl 3CP inhibitors where the identical substitution resulted in slightly improved activity (1.8-fold vs. HRV-14 3CP).[10] However, since the (4-F,*N*-Me)Phe-containing molecules exhibited somewhat improved *in vitro* stability relative to the corresponding non-fluorinated inhibitors, this functional group was retained in several additional compounds.[22] Thus, combination of P_3 amino acids containing aromatic side chains with the N-terminal 5-methylisoxazole-3-carboxamide and P_2 (4-F,*N*-Me)Phe moieties also afforded several active 3CP inhibitors and antiviral agents (compounds **10-12**).

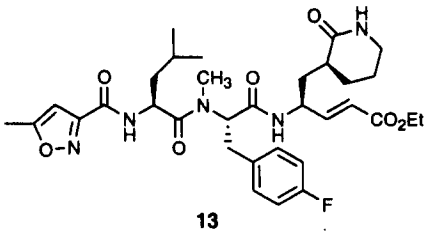
Table 3.



Compd.	R	X	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)[a]	EC_{50} (μM)[a]
8	$CH_2CH(CH_3)_2$	H	108,000	0.14
9	$CH_2CH(CH_3)_2$	F	59,300	0.40
10	CH_2(1-Naphthyl)	F	95,800	0.20
11	CH_2(2-Naphthyl)	F	144,000	0.16
12[b]	CH_2(4-Imidazole)	F	66,000	1.8

[a]Serotype-14; [b]TFA salt.

Finally, we also prepared a P_2 *N*-methylamide-containing inhibitor which included a P_1-lactam moiety in lieu of the glutamine-derived fragment (compound **13**). As expected from previous SAR studies,[23] this modification significantly improved both anti-3CP and antiviral activity relative to the corresponding glutamine-containing inhibitor (compare **9** with **13**). Although not quite as potent as optimized tripeptidyl and ketomethylene-derived molecules,[18,23] compound **13** demonstrates that *N*-methylamide-containing tripeptide Michael acceptors can function as both highly active 3CP inhibitors and effective *in vitro* antirhinoviral agents.

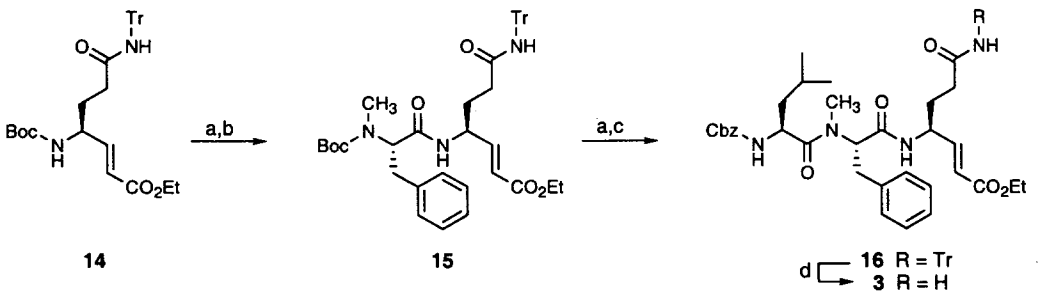


13

$k_{obs}/[I]$ = 610,000 $M^{-1}s^{-1}$
EC_{50} = 0.03 μM
(Serotype-14)

The 3CP inhibitors described in this work were prepared by methods analogous to those utilized previously to synthesize related non-methylated tripeptidyl compounds.[9,10] A typical preparation is illustrated by the synthesis of inhibitor **3** (Scheme 1).[24-26] In contrast to the highly crystalline non-methylated inhibitors,[9,10] the *N*-methyl amino acid-containing molecules typically exist as oils or waxes and required flash column chromatography for purification.

Scheme 1.



14 15 16 R = Tr, 3 R = H

Reagents and conditions (Tr = CPh_3): (a) HCl, 1,4-dioxane, 23 °C, 1 h; (b) Boc-(*N*-Me)Phe-OH, EDC, HOBt, NMM, CH_2Cl_2, 23 °C, 18 h, 46%; (c) Cbz-Leu-OH, EDC, HOBt, NMM, CH_2Cl_2, 23 °C, 18 h, 36%; (d) TFA, CH_2Cl_2, $(iPr)_3SiH$, 23 °C, 73%.

In summary, tripeptide-derived molecules which incorporate C-terminal Michael acceptor moieties and P_2 *N*-methyl amino acid residues are shown to be potent irreversible inhibitors of the human rhinovirus 3C protease and highly active *in vitro* antirhinoviral agents.

Acknowledgments. We are grateful for many helpful discussions throughout the course of this work with Prof. Larry Overman and Dr. Steven Bender. We also thank Dr. Kim Albizati, Dr. Srinivasan Babu, Terence Moran, Ray Dagnino, and Miguel Pagan for the large-scale preparation of several intermediates.

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13. The nomenclature used for describing the individual amino acid residues of a peptide substrate (P_2, P_1, P_1', P_2', etc.) and the corresponding enzyme subsites (S_2, S_1, S_1', S_2', etc.) is described in: Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.

14. Enzyme assays were performed as described in ref. 9.

15. All amino acids described in this work are L isomers.

16. Antiviral assays were performed using H1 HeLa cells as described in ref. 9. The antirhinoviral properties of certain 3CP inhibitors were determined against several additional HRV serotypes in cell culture. In general, compounds which displayed activity against HRV serotype 14 exhibited related, albeit less potent (2 to 10-fold), antirhinoviral properties when tested against serotypes 1A, 2, and 10.

17. The toxicities of the molecules described in this work were determined using H1 HeLa cells as described in ref. 9. All compounds were non-toxic when tested to the 10 μM level.

18. Dragovich, P. S.; Prins, T. J.; Zhou, R.; Fuhrman, S. A.; Patick, A. K.; Matthews, D. A.; Ford, C. E.; Meador, J. W., III; Ferre, R. A.; Worland, S. T. *J. Med. Chem.* **1999**, *42*, 1203.

19. The 3CP residue with which the P_2-P_3 amide NH of **1** interacts (Ser-128) is solvent exposed and known to be conformationally mobile (refs. 4, 9, and 18). Analysis of the HRV-2 3CP-**3** X-ray crystal structure revealed displacement of this residue relative to its observed location in the HRV-2 3CP-**1** complex (ref. 9) to allow for binding of the P_2 *N*-methylamide moiety. Otherwise, the *N*-methylamide-containing inhibitor **3** bound to 3CP in a manner that was nearly identical to that noted for the non-methylated compound **1** (D. Matthews, unpublished results).

20. The P_3 Val for Leu substitution resulted in a 2.5-fold improvement in anti-3CP activity (HRV-14) when applied to non-methylated tripeptidyl inhibitors. See ref. 10.

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24. HATU [*O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate] was also utilized as a coupling reagent in the course of several syntheses. See: Carpino, L. A.; El-Faham, A. *J. Org. Chem.* **1995**, *60*, 3561.

25. The trityl-protected (*N*-Me)glutamine derivative required for the synthesis of compound **2** was prepared from Fmoc-(γ-tBu,*N*-Me)Glu-OH by the following sequence: (i) isobutyl chloroformate, NMM, $HN(CH_3)OCH_3$•HCl; (ii) $LiAlH_4$; (iii) $(EtO)_2POCH_2CO_2Et$, $NaN(TMS)_2$; (iv) TFA; (v) $SOCl_2$, NH_4OH; (vi) $HOCPh_3$, Ac_2O.

26. The Boc-protected, lactam-containing intermediate required for the preparation of compound **13** was synthesized according to the published procedure (ref. 23).