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SMALL PEPTIDIC ALDEHYDE INHIBITORS OF HUMAN RHINOVIRUS 3C PROTEASE

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Abstract: Small peptide aldehydes were designed to mimic the preferred substrate requirements for the human rhinovirus 3C protease. Di- and tripeptide aldehydes containing a methionine sulfone as a P_1 surrogate for glutamine show low micromolar enzyme inhibitory and antiviral tissue culture activity. LY338387, obtained in a short and efficient synthesis, appears to validate the protease as a therapeutic target.

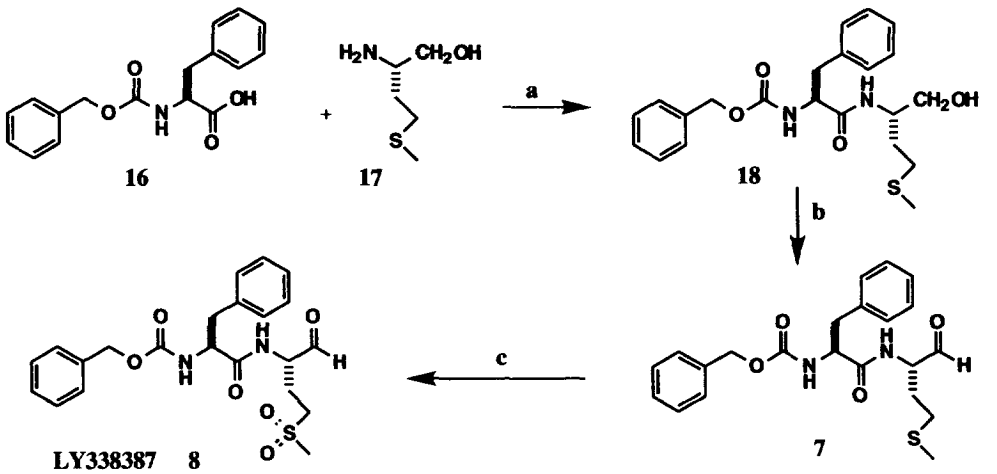
The human rhinoviruses (HRVs) are believed to be the major cause of the common cold.[1] Their positive strand RNA genome is translated directly into a polyprotein that is cleaved by the 3C proteinase ($3C^{pro}$), or its precursor 3CD, resulting in mature capsid and viral proteins that ultimately assemble into infectious virions.[2] The $3C^{pro}$ was recently shown to be a cysteine protease in a trypsin-like fold.[3] Because $3C^{pro}$ makes highly specific cleavages that are essential to viral replication, it would appear to be an attractive target for antiviral therapy.

$3C^{pro}$ makes its cleavages primarily at the Gln-/-Gly bond. Colonno and coworkers determined the hexapeptide Thr-Leu-Phe-Gln-/-Gly-Pro to be the minimally effective substrate at the 2C-3A cleavage site.[4] Relatively few inhibitors of HRV $3C^{pro}$ have been reported and none have demonstrated low micromolar antiviral activity[5-8] Recently two tetrapeptide aldehydes, based upon the above sequence requirement (P_4-P_1), were reported to be good enzyme inhibitors.[9,10] However, the combination of the P_1 glutamine and aldehyde functionalities presents an interesting dilemma. In the case of the Kaldor et al. inhibitor **15** (N-Boc-Leu-Val-Phe-Gln-CHO) the free glutamine cyclizes on the aldehyde to provide the preferred glutaminal tautomer.[10] The compound retains good enzyme inhibition (IC_{50} = 0.6 μM) but lacks good tissue culture activity (IC_{50} = 400 μM).[11] The Malcolm group tetrapeptide (N-Ac-Ala-Ala-Ala-Gln (NMe_2)-CHO) protects the glutamine as the dimethyl amide and also retains good enzyme inhibition even though the sequence is obviously not optimized for HRV $3C^{pro}$ (K_i = 0.34 μM).[9] These results were consistent with their hypothesis that "an uncharged δ-carbonyl oxygen is required in the P_1 position of the substrate for efficient recognition by the picornaviral enzymes." Unfortunately, no antiviral data was reported for this inhibitor.

We thought that a P_1 Met (SO_2) might effectively mimic the glutamine and simplify the above synthetic limitations. Compound **2** (Table 1) is a commercially available tripeptide calpain II inhibitor that we imagined could test this isostere concept by a simple one-step oxidation of the methionine. We were encouraged that this compound **3** (LY335230) showed good enzyme inhibition (K_i = 0.49 μM),[12] potent in vitro translation inhibition,[13,14] and apparent antiviral activity (Table 1).[15] However, to improve activity we sought to more closely mimic the substrate requirements of HRV-14 $3C^{pro}$. Tsujinaka and coworkers reported the improved cell penetration for the Z-protected dipeptide calpain inhibitor calpeptin (**4**).[16] Others have also noted Z-protected dipeptide aldehydes to be good inhibitors of cathepsin L[17] and HIV protease.[18] These observations led us to synthesize the dipeptide LY338387 (**8**) incorporating a P_2 Phe substituent (Scheme 1).

In an efficient, straightforward synthesis Cbz-protected phenylalanine was coupled to L-methioninol in 55% yield. The alcohol was selectively oxidized under nonracemizing conditions to the aldehyde **7** with sulfur trioxide pyridine complex (10 min, room temperature). The methionine was then oxidized to the sulfone using Oxone®.[19] LY338387 was a potent reversible inhibitor of $3C^{pro}$ with a $K_i = 0.47$ μM. We also observed good tissue culture activity (Table 1, $IC_{50} = 3.4$ μM) without cytotoxicity ($TC_{50} > 224$ μM). Further evaluation of **3** and **8**, including an in vitro translation assay, will be reported in due course.[14] The precursor sulfide **7** also showed good antiviral activity but with a poorer therapeutic index (TI) (see Table 1 footnote *c*). We cannot rule out the possibility that some in situ oxidation of the methionine is giving rise to activities similar to the methionine sulfone analogs.

SCHEME 1. Synthesis of LY338387



(a) CDI, THF, 55%; (b) Pyridine-SO_3, Et_3N, DMSO, 94%; (c) Oxone®, MeOH, H_2O, 85%.

A short SAR was then initiated to examine the effect of changing the P_2 Phe substituent. Compounds **6**, **10**, **12**, and **13** were synthesized in the same general manner as shown in Scheme 1. Substitution of Leu for Phe (**6**) caused a 12-fold loss in antiviral activity. Substitution of homophenylalanine for Phe (**12**) caused a 24-fold loss in antiviral activity while the D-Phe analog (**13**) was completely devoid of antiviral activity.

Replacement of the Cbz protecting group of LY338387 with the Boc group resulted in a weak enzyme inhibitor (**10**, $IC_{50} = 60$ μM) and a 21-fold loss in antiviral activity. Finally, we wanted to make the Z-protected dipeptide analog in the glutamine aldehyde series to see if the antiviral activity would be improved. Kaldor et al. reported that the Boc protected dipeptide (N-Boc-Phe-Gln-CHO) was a poor enzyme inhibitor ($IC_{50} > 80$ μM).[10] We followed their reported procedure for making the glutarimide of glutamine, which was coupled to N-Cbz-Phe, followed by selective reduction of one of the glutarimide carbonyls with sodium borohydride to give compound **14**. We were pleased to find an almost 10-fold improvement in antiviral activity over the tetrapeptide **15** even though the compound was less potent as an enzyme inhibitor ($IC_{75} = 60$ μM).

In summary, we are the first to report dipeptide aldehydes which give low micromolar antirhinoviral (HRV-14) activity in tissue culture. LY338387 can be rapidly synthesized and appears to be a suitable positive control for a high throughput 3C protease inhibitor assay.[20] These results suggest the 3C protease could be a useful therapeutic target for antiviral therapy.

TABLE 1. Antiviral Activities of Peptide Aldehydes

Compound	IC_{50} (μM)[a]	TC_{50} (μM)[b,c]
1 N-Ac-Leu-Leu-Arg-CHO (Leupeptin)	>224	>224
2 N-Ac-Leu-Leu-Met-CHO	23.7	37.1
3 N-Ac-Leu-Leu-Met (SO_2)-CHO (LY335230)	81.9	>224
4 N-Cbz-Leu-Nle-CHO (Calpeptin)	17.7	22.1
5 N-Cbz-Leu-Met-CHO	46.8	83.0
6 N-Cbz-Leu-Met (SO_2)-CHO	39.3	>224
7 N-Cbz-Phe-Met-CHO	9.7	42.2
8 N-Cbz-Phe-Met (SO_2)-CHO (LY338387)	3.4	>224
9 N-Boc-Phe-Met-CHO	84.1	65.4
10 N-Boc-Phe-Met (SO_2)-CHO	71.3	147.6
11 N-Cbz-HPhe-Met-CHO[d]	20.3	13.5
12 N-Cbz-HPhe-Met (SO_2)-CHO	81.2	161.5
13 N-Cbz-D-Phe-Met (SO_2)-CHO	>224	>224
14 N-Cbz-Phe-Gln-CHO	42.0	>224
15 N-Boc-Val-Leu-Phe-Gln-CHO	400[11]	

[a]Antiviral activity measured by 48 h plaque reduction assay (HRV-14, HeLa cells).[15] [b]Cytotoxic effect measured by XTT assay.[13] [c]TI, therapeutic index (defined as TC_{50}/IC_{50}). By convention, true antiviral activity requires a TI ≥ 10. [d]HPhe = Homophenylalanine.

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11. Reference 10 gives the tissue culture IC_{50} of compound **15** as 500 μM in the reference and notes section, whereas reference 13 gives an IC_{50} = 300 μM. We have used an average of these reports.

12. Inhibition of purified 3C protease (Birch, G. M.; Black, T.; Malcolm, S. K.; Lai, M. T.; Zimmerman, R. E.; Jaskunas, S. R. *Protein Expr. Purif.* **1995**, *6*, 609) measured with a fluorescence assay using the substrate Anthranyl-TLFQGPV(pNO_2Phe)K.[14]

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15. Plaque reduction assay for HRV14: Confluent monolayers of HeLa cells were infected using 100 pfu per plate. After an adsorption period of 60 min the inocula were replaced by 1.5 mL of a maintenance medium overlay containing 0.5% agarose and supplemented with various concentrations of test compound. The plates were incubated at 34 °C for 48 h and the infected monolayers were then fixed with buffered 10% formalin in H_2O and then stained with crystal violet after removal of the overlay. The mean plaque number was calculated from a duplicate series of counts, converted to a percentage of untreated controls and plotted against the $\log_{10}$ concentration of test compound. This data was used to calculate the IC_{50}.

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19. All compounds gave satisfactory analytical or exact mass and spectral data. ^{1}H NMR data for LY338387: ($CDCl_3$, 300 MHz) δ 9.31 (s, 0.5H), 7.42-7.18 (m, 10H), 7.06 (d, 1H, J = 7.3 Hz), 5.53 (d, 1H, J = 6.4 Hz), 5.05 (s, 2H), 4.45 (m, 1H), 4.32 (m, 1H), 3.10-2.91 (m, 4H), 2.83 (s, 3H), 2.40 (m, 1H), 2.02 (m, 1H). Based upon the integration of the aldehyde proton the compound appears 50% hydrated. TLC one spot R_f = 0.65 (90:10 CH_2Cl_2:MeOH). FABMS (M+H) : 447. Anal. calcd for $C_{22}H_{26}N_2O_6S$: C, 59.18; H, 5.87; N, 6.27; Found: C, 58.98; N, 6.06; N, 6.00.

20. Steve Kahl, Rob Johnson and Q. May Wang (Lilly Research Labs) private communication.

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