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INHIBITION OF HUMAN RHINOVIRUS 3C PROTEASE BY HOMOPHTHALIMIDES

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Abstract: Homophthalimides **2a** and **3a** were found to be inhibitors of Rhinovirus 3C protease through a blind screening effort. SAR studies resulted in compound **3g**, which exhibited improved enzyme inhibition, in addition to whole cell antiviral activity. Molecular modeling studies suggest a preferred enzyme/inhibitor interaction, and LC/MS experiments confirmed tight/covalent binding of **3g** to the enzyme.
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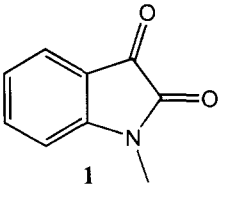
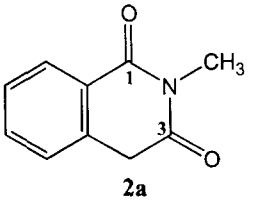
Human rhinoviruses (HRVs) comprise more than a hundred distinct serotypes and are the major etiological agents of the common cold in humans.[1] These small plus-stranded RNA viruses translate their genetic information into a single large viral polyprotein. This polyprotein is subsequently cleaved by virally encoded proteases, to generate the mature viral enzymes and structural proteins.[2,3] It is believed that the majority of the maturation cleavages are performed by the HRV-14 3C protease ($3C^{pro}$).[2,3] Though $3C^{pro}$ is a cysteine protease (contains an active site cysteine nucleophile), it is structurally more closely related to the chymotrypsin family of serine proteases, rather than typical cysteine proteases.[4] Evidence has accumulated that the viral $3C^{pro}$ is not only an important protease, but also an RNA-binding protein implicated in viral replication.[3] $3C^{pro}$ plays an important role in viral infection, and possesses a unique protein structure, therefore, it may be an ideal target for drug development.

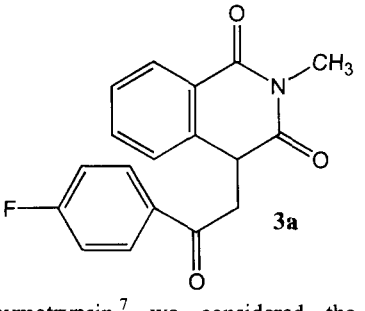
A blind screening program was initiated in an effort to identify inhibitors of $3C^{pro}$. The activity of the screen hits was confirmed through an in vitro translation assay.[5] Two of the more interesting hits were the isatin **1** and the homophthalimides **2a** and **3a**. As isatins are known to have activity against several different RNA



1



2a



3a

dependent viral polymerases,[6] in addition to activity against chymotrypsin,[7] we considered the homophthalimides to be a more intriguing lead. The Agouron group has recently reported on their efforts to optimize the $3C^{pro}$ activity of the isatins.[8] An attractive feature of the homophthalimides is the possibility of synthetic modification at three sites (aromatic ring, benzylic methylene, N-substitution), thus allowing for the potential of a more structurally diverse SAR. Several other groups have reported inhibitors of $3C^{pro}$.[9] Herein, we report on our efforts to improve the $3C^{pro}$ inhibitory activity of the homophthalimides.

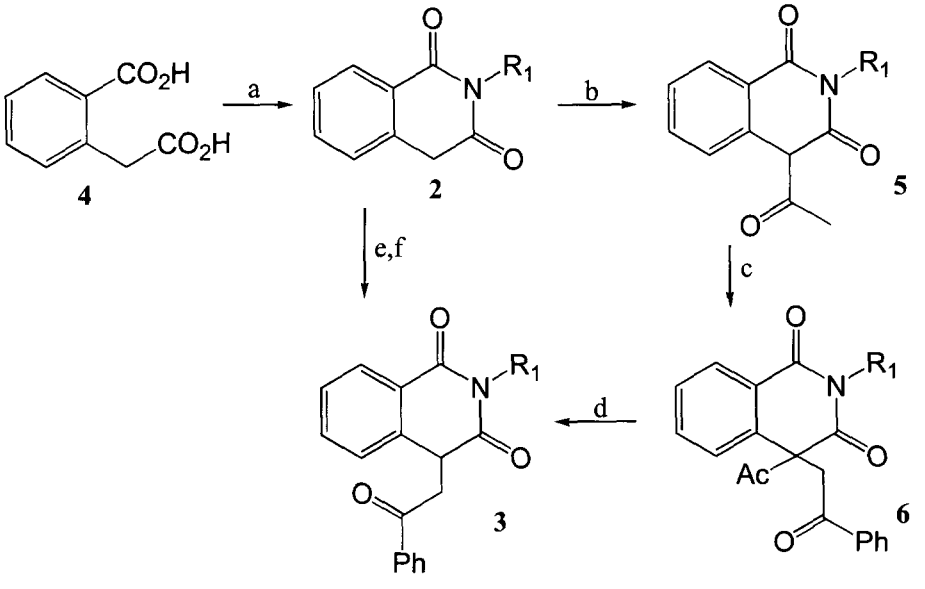
Chemistry

The unsubstituted homophthalimides **2** were synthesized via the condensation of homophthalic acid (**4**) and the corresponding primary amines (Scheme 1). Thus, refluxing the bis–acid **4** and an amine, in *o*-xylene, with concomitant removal of water via a Dean–Stark trap provided **2** in good yields. Initially, conversion of **2** to monoalkylated products, **3**, proved problematic. Generation of the homophthalimide anion (1.1 equiv of LDA at -78 °C), followed by addition to a solution of the bromoacetophenone at -78 °C, produced only bis-alkylated products.

One route to monoalkylated compounds, **3**, involved initial monoacetylation with DMAP, pyridine, and acetic anhydride, to give **5** in high yields. Alkylation of the active methylene gave **6**, followed by base catalyzed deacetylation, provided the desired monoalkylated homophthalimide **3**. Ultimately, a direct route to alkylation was found which produced acceptable yields of monoalkylated products. This involved treatment of the homophthalimide **2** with 1.1 equiv of LDA at -78 °C, followed by cannulation of the -78 °C anion solution into a 0 °C solution of the bromoacetophenone reagent, to provide **3c** and **3h**, in 65–75% yields.[10]

The sulfides corresponding to **3g** and **3i** were synthesized in the same fashion, followed by *m*-CPBA oxidation (2.2 equiv, 0 °C) to produce the desired sulfones, in quantitative yields. Sulfoxide **2f** was made by oxidation of **2e** with a single equivalent of *m*-CPBA, at -78 °C, also in quantitative yield.

Scheme 1. Synthesis of Homophthalimides



(a) H_2NR_1, refluxing *o*-xylene; (b) Ac_2O, pyridine, DMAP, CH_2Cl_2; (c) $BrCH_2COPh$, K_2CO_3, CH_2Cl_2; (d) NaOMe, MeOH; (e) 1.1 equiv LDA, **2**, THF, -78 °C, 30 min; (f) cannulate -78 °C solution from (e) into 0 °C solution of $BrCH_2COPh$.

Results and Discussion

$3C^{pro}$ hydrolyzes its natural substrate primarily between Gln-Gly residues (six of eight natural cleavage sites contain this motif). Three of these sites contain a Gln-Gly-Pro motif. In an attempt to more closely mimic the natural substrate, we replaced the N-methyl group of the lead structure **2a** with glutamine-like substituents (e.g., $R_1 = CH_2CH_2COR$). As our group had previously reported that a methionine sulfone residue can serve as an effective glutamine mimic[11] in $3C^{pro}$ inhibitors, we also prepared compounds where $R_1 = CH_2CH_2S(O)_nCH_3$ (n = 0, 1, 2). As can be seen in Table 1 compounds **2c-g** have similar inhibitory activity to the lead compound **2a**, whereas carboxylic acid containing compound **2b** was devoid of activity. Likewise, in the alkylated series **3a**, **c**, **g-i**, the R_1 substituent appeared to have minimal effect on enzyme inhibition. In all cases the acetophenone substituted compounds exhibited superior enzyme inhibition relative to unsubstituted compounds. Two of the compounds, **3g** and **3i**, exhibited somewhat better enzyme inhibitory activity, relative to **3a**. It is also interesting to note that sulfone **3i** appears to be a better enzyme inhibitor than the sulfide **3h**.

Table 1. HRV-14 $3C^{pro}$ Enzyme inhibitory activity

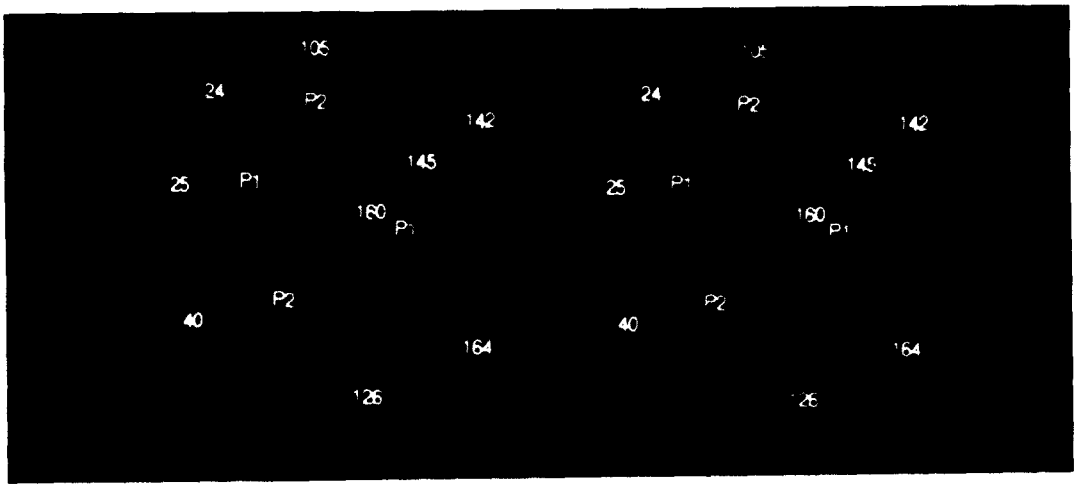
Compound	R^1	R^2	% Inhibition[a]	IC_{50} (μM)[a]
2a	$-CH_3$	-H	14	
2b	$-(CH_2)_2CO_2H$	-H	0	
2c	$-(CH_2)_2CO_2Et$	-H	13	
2d	$-(CH_2)_2CONH_2$	-H	11	
2e	$-(CH_2)_2SMe$	-H	13	
2f	$-(CH_2)_2SOMe$	-H	19	
2g	$-(CH_2)_2SO_2Me$	-H	6	
2h	$-(CH_2)_3SMe$	-H	16	
3a	$-CH_3$	$-CH_2COPh(p\text{-}F)$	72	41.1
3c	$-(CH_2)_2CO_2Et$	$-CH_2COPh$	71	55.3
3g	$-(CH_2)_2SO_2Me$	$-CH_2COPh$	100	22.1
3h	$-(CH_2)_3SMe$	$-CH_2COPh$	35	130.6
3i	$-(CH_2)_3SO_2Me$	$-CH_2COPh$	70	25.0
5c	$-(CH_2)_2CO_2Et$	-COMe	8	

[a]Measured using a colorimetric peptide assay.[12] Percent inhibition determined at 25 μg/mL.

In order to gain a better understanding of the SAR of the homophthalimide series of compounds we performed molecular modeling studies[13] of complexes with human rhinovirus-14 $3C^{pro}$. The calculations are based on NOE distances from protein multidimensional NMR studies of H_2N-Leu-Phe-Gln-CHO peptide complexed with the proteinase (A. D. Kline, unpublished results). A simple interaction energy[14] was used to correlate energetics with enzyme activities. The interaction energies for complexes of $3C^{pro}$ with homophthalimides **2c**, **3a**, and **3g** are -43.8, -55.9, and -80.4 kcal/mol in general agreement with the results presented in Table 1. The active site environment of $3C^{pro}$ with **3g** is shown in Figure 1. The C1 carbonyl group is positioned for interaction with the oxyanion hole while the N-alkyl group is situated within the P1 active site. This arrangement situates the acetophenone group in a large channel at the P1′ and P2′ positions

which provides ample space for the aromatic ring to nestle and interact with the Phe-25. This model also assumes that the C3 (non-benzylic) carbonyl would be the most likely site of nucleophilic attack by the active site cysteine.

Figure 1. Stereo representation of a model for **3g** bound to $3C^{pro}$. Salient residues are numbered and peptidic sites are labeled.



The interaction of several homophthalimides, **3c**, **3g**, **3i**, and **5**, with $3C^{pro}$ was also studied by mass spectroscopy.[15] Each of these compounds, with the exception of **5**, produced a peak corresponding to the mass of the inhibitor plus enzyme (M^+ = MW + 19,999) as well as a peak for the free enzyme (M^+ = 19,999). This observation suggests inhibitors **3c**, **3g**, and **3i** are tightly (if not covalently) bound to $3C^{pro}$ in a ratio of 1:1 (inhibitor:enzyme). To determine which site(s) of the protease had been modified by the inhibitors, tryptic peptide mapping of the $3C^{pro}$ pretreated with DMSO or with **3g** and **3i** was performed. Their HPLC profiles were identical, with the exception that the peak which is known to contain the active site cysteine residue, disappeared in the inhibitor modified protease samples (data not shown). This finding indicates that homophthalimides **3g** and **3i** inhibit $3C^{pro}$ through an interaction with the active site cysteine residue.

Several of the active compounds were tested for whole cell antiviral activity in a one-cycle viral replication assay (Table 2). It appeared that at least one compound, **3c**, exhibited true antiviral activity. To the best of our knowledge, this is the first nonpeptidic $3C^{pro}$ inhibitor to show antiviral activity against HRV-14, with a TI $\geq$ 10. It was certainly of note that the inherent whole cell activity of **3c** exceeds its $3C^{pro}$ enzyme inhibitory activity. Preliminary experiments indicate that homophthalimides may also inhibit the other HRV cysteine protease, 2A (Q. May Wang, unpublished results). The results of these studies will be communicated in due course.

Table 2. Antiviral activity

Compound	IC_{50} (μM)[a]	TC_{50} (μM)[b]	TI[c]
3a	>10	>100	
3c	6.0	>100	16.7
3g	8.5	70.50	8.3
3h	>10	62.97	
3j	>10	>100	

[a]One-cycle viral replication assay.[16] [b]Cytotoxic effect measured by XTT assay.[5] [c]TI, therapeutic index (defined as TC_{50}/IC_{50}). By convention, true antiviral activity requires a TI ≥ 10.

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10. All compounds gave satisfactory spectral data (C, H, N elemental analysis, ^{1}H NMR, MS).

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12. $3C^{pro}$ assay: Purified HRV-14 3C protease (0.4 μM) was incubated with the indicated compound at different concentrations in a reaction mixture containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 10% DMSO for 6 h at room temperature. The cleavage reaction was initiated by addition of 250 μM chromogenic peptide substrate with a sequence of EALFQ-*p*-nitroaniline. Absorbance at 405 nm was taken at the time point of 30 min. At least seven different concentrations were used for each inhibitor to generate IC_{50} values. Percentage of 3C activity inhibition was determined under the conditions described above at a fixed concentration of 25 μg/mL. The chromogenic substrate used in this assay (EALFQ-*p*-nitroaniline) is different from the substrate used in the blind screening effort (RAEFQ/GPYDE). The apparent relative affinity of these two substrates for $3C^{pro}$ accounts for the finding that weak inhibitors such as **2a** appeared active in the blind screen.

13. Molecular modeling experiments were performed with the CHARMM program (Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. *J. Comp. Chem.* **1983**, *4*, 187). Random conformations of the inhibitors were docked within the active site and the complexes were subsequently subjected to repeated short term molecular dynamics and energy minimization. The resulting structures were energy minimized to zero energy gradient and analyzed.

14. A method for predicting in vitro enzyme activity of HIV-1 proteinase complexes has been reported. Holloway, M. K.; Wai, J. M.; Halgren, T. A.; Fitzgerald, P. M. D.; Vaccs, J. P.; Dorsey, B. D.; Levin, R. B.; Thompson, W. J.; Chen, L. J.; deSolms, S. J.; Gaffin, N.; Ghosh, A. K.; Giuiliani, E. A.; Graham, S. L.; Guare, J. P.; Hungate, R. W.; Lyle, T. A.; Sanders, W. M.; Tucker, T. J.; Wiggins, M.; Wiscount, C. M.; Woltersdorf, O. W.; Young, S. D.; Drake, P. L.; Zugay, J. A. *J. Med. Chem.* **1995**, *38*, 305; and references therein.

15. Mass spectroscopy analysis: Electrospray ionization mass spectrometry was conducted using a PESciex API III triple stage quadrupole mass spectrometer. The instrument was operated in the positive ion detection mode, with an IonSpray voltage of 3500V. Purified $3C^{pro}$ (20 μM) was incubated for 6 h with the specified compounds (125–130 μM) or DMSO as a control. The treated enzyme samples were then diluted 1:1 with 1% acetic acid in acetonitrile (v/v) and continuously infused into the interface at a rate of 10–20 mL/min. using a syringe pump. MS spectra were collected over a range of 1000–2000 u at 0.1 u intervals with a dwell time of 1 msec per interval. A total of 10–20 scans were averaged to yield the final spectrum.

16. A monolayer of HeLa cells was innoculated with HRV-14 at a multiplicity of infection of 1 PFU per cell and adsorption allowed to take place for 30 min at room temperature. The infected cells were incubated for 8 h at 35 °C with medium and cell lysate was sonicated for 10 min and centrifugated at 4,000 x *g* for 15 min at 4 °C. The virus in the supernatant was then quantitated by use of the plaque forming assay in HeLa cells as described previously (Tyms, A. S.; Scamans, E. M.; Naim, H. M. *J. Antimicrob. Chem.* **1981**, *8*, 65). To estimate the effective concentrations for 50% inhibition of HRV-14 replication (IC_{50}), percent inhibition of the virus yield was plotted against the logarithm of the concentration of the compound.

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