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THE INHIBITION OF HUMAN CYTOMEGALOVIRUS (hCMV) PROTEASE BY HYDROXYLAMINE DERIVATIVES

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Abstract: Aryl hydroxylamine derivatives have been synthesised that are some of the most potent inhibitors of hCMV protease prepared to date (IC_{50} 14-60 nM). Mass spectrometry studies indicate that oxazinone derived hydroxylamines inhibit the enzyme by acylation of Ser^{132} whereas non-oxazinone derived hydroxylamines appear to inhibit via formation of a sulfinanilide at Cys^{138} .

Cytomegalovirus (CMV) is a member of the β-herpes virus family and is carried by a large percentage of the world population. In normal circumstances the virus is not life-threatening but in immunocompromised situations (AIDS sufferers, neonates, post-transplantation patients) it poses a high risk of mortality and morbidity.[1] Current therapy relies upon drugs which have a narrow therapeutic index and are associated with a number of unpleasant side effects, hence there is a major unmet need for remedies which are both efficacious and safe to combat these infections.[2] Recently, specific herpes virus proteases, the assemblins, have been discovered which are essential to the viral capsid maturation process and their inhibition offers a potential treatment for CMV and other herpes virus infections.[3] The protease target is attractive for a number of reasons *viz* inhibitors might be expected to prevent the formation of infectious viral particles and thus exert an anti-viral action, the capsid assembly process is not mimicked in the mammalian environment and the protease is of unusual structure,[4] relying on a novel catalytic triad for activity (Ser^{132}, His^{63}, His^{157} in the case of hCMV), which has no homology with mammalian proteases. Virus-host selectivity would thus appear attainable.

Recent reports describe a number of mechanism-based inhibitors of the β-herpes viruses which include spiro-oxazolones and imidazolones,[5] β-lactams,[6] benz-[7] and thieno[8]-oxazinones. The mechanism of inhibition of compounds derived from the latter template is thought[8] to derive from an acyl-enzyme complex formed by attack of the active site serine (Ser^{132}) at the oxazinone carbonyl, followed by ring opening. We present here our studies with a number of hydroxylamine derivatives that were prepared as part of a programme to investigate the incorporation of moieties with H-bonding potential into compounds having a thieno-oxazinone template. Additional H-bonding interactions were sought in an attempt to augment potency vs the enzyme.

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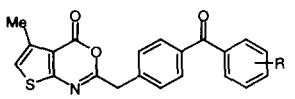
Chemistry

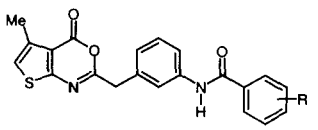
The benzophenone derivatives **A** (where R = H, 3-NO_2, 4-NO_2, 3-CHO, 4-CH_2OTBDMS) were prepared either by coupling of 2-amino-4-methylthiophene-3-carboxylic acid with the appropriate substituted phenylacetic acid followed by dehydrative cyclisation (EDC, HOBT, DMF) or by similar coupling of the same thiophene with 4-iodophenylacetic acid followed by palladium catalysed carbonylation with the appropriate boronic acid.[9] Reduction of the nitrobenzophenone **2** (H_2, 10% Pd/C, EtOAc, 5 h, RT) gave the hydroxylamine **3** as the main product (58%) together with the aniline **7** (7%). Acetylation (Ac_2O, DCM, RT, 18 h) of the hydroxylamine **3** gave a mixture of the N-acetyl **8** and N,O-*bis*-acetyl **9** derivatives. Reduction ($NaBH(OAc)_3$, toluene, 80°C, 18 h) of the aldehyde precursor (**A**, R = 3-CHO) gave the hydroxymethyl derivative **5** and cleavage (HF-pyridine, DCM, RT) of the silylated precursor (**A**, R = 4-CH_2OTBDMS) gave the hydroxymethyl derivative **6**.

EDC-mediated coupling and cyclisation of the same aminothiophene with 3-nitrophenylacetic acid followed by reduction gave the 2-(3-aminobenzyl) substituted oxazinone which served as the common precursor to the amide series **B**. Alternatively, this was prepared by reaction of the thiophene with N-Boc-protected 3-aminophenylacetic acid followed by deprotection (TMSI). EDC-mediated coupling of this amine with 3- and 4-nitrobenzoic acids followed by reduction using the same conditions as described above gave the hydroxylamines **11** and **12**. Similar coupling with 3- and 4-N-Boc-protected hydrazinobenzoic acids followed by deprotection (TMSI) gave the hydrazines **13** and **14** and coupling with 3-(O-methyl-N-Boc-hydroxylamino) benzoic acid followed by deprotection (TMSI) gave the O-methylated hydroxylamine **15**.

Table 1



A



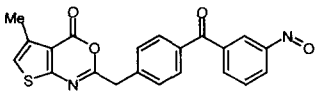
B

A	R	CMV IC_{50} uM	A	R	CMV IC_{50} uM	B	R	CMV IC_{50} uM	B	R	CMV IC_{50} uM
1	H	0.8	6	4-CH_2OH	0.94	10	H	1.5	13	3-$NHNH_2$	3.8
2	3-NO_2	3.5	7	3-NH_2	1	11	3-NHOH	0.045	14	4-$NHNH_2$	2.4
3	3-NHOH	0.019	8	3-N(Ac)OH	1.1	12	4-NHOH	0.061	15	3-NHOMe	2.2
4	4-NHOH	0.014	9	3-N(Ac)OAc	1.4						
5	3-CH_2OH	1.1									

The hydroxylamine **3**, its 4-isomer **4** and the analogues **11** and **12** from the amide linked series are significantly more potent inhibitors of hCMV than the unsubstituted derivatives **1** and **10**. This high level of potency is lost in the aniline **7**, hydroxymethyl **5** and **6** and hydrazine **13** and **14** derivatives. Acetylation and

O-methylation of the hydroxylamine (compounds **8**, **9** and **15** respectively) resulted in a 50-100 fold reduction in potency. These data would appear to indicate a specific effect of the hydroxylamine moiety itself.

Stability studies on the hydroxylamine **3**, performed in the assay medium, demonstrated degradation to nitroso (**16**) and azoxy (**17**) derivatives, consistent with literature reports on hydroxylamine stability.[10,11] Hence these compounds were prepared to evaluate their role in the activity formally shown by the hydroxylamine. The nitroso derivative (prepared by treating **3** with diethyl azodicarboxylate, DCM, RT, 18 h) was a potent inhibitor (IC_{50} 0.055 uM), whereas the azoxy compound (**3** + **16**, $CHCl_3$, RT, 17 days) was inactive (IC_{50} >33 uM).



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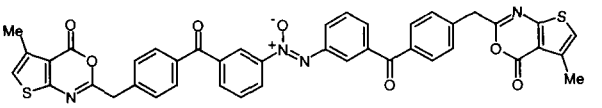

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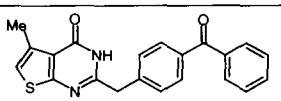
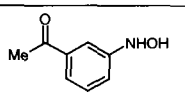
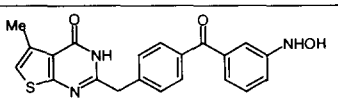
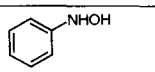
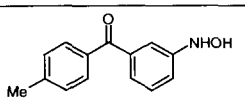
To probe further the activity of the hydroxylamine derivatives the corresponding thienopyrimidinone **19** was prepared (**2** + NH_3 followed by H_2, 10% Pd/C). The potency of this compound (Table 2) was 100 fold less than that of **3** but, nevertheless, was equipotent with some of the non-hydroxylamine oxazinone derivatives of Table 1. This was unusual for a template generally devoid of inhibitory activity vs the β-herpes viruses (cf **18**), and the activity of the methylbenzophenone **20** confirmed that the thienopyrimidinone moiety itself offered little additional binding compared to that of a benzophenone hydroxylamine. Sequential removal of further fragments established that the phenylhydroxylamine unit was virtually solely responsible for the inhibition seen with these compounds, although hydroxylamine itself was inactive under these conditions.

Table 2

#	Structure	CMV IC_{50} uM	#	Structure	CMV IC_{50} uM
18		>33	**21**		11
19		2	**22**		9
20		3.7	**23**	$NH_2OH.HCl$	>33

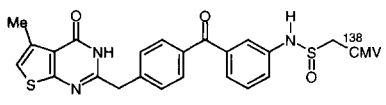
To elucidate whether **19** was intrinsically active or if oxidation to the nitroso derivative (or an intermediate short-lived species on the degradation pathway) was responsible for the activity, the compound was evaluated in the presence of dithiothreitol (DTT). No inhibition of the protease was observed - presumably any oxidative degradation of the compound being arrested under these conditions. This result would suggest

that the increased potency shown by **19** and, by implication those hydroxylamines derived from the thienoxazinone template, is not manifested by the hydroxylamine moiety *per se*. (The control experiment with a thienoxazinone was not carried out since ring opening of oxazinones was known to occur with DTT – data not shown.)

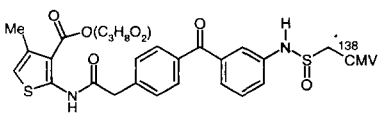
Mass Spectrometry

To aid interpretation of the inhibition resulting from the hydroxylamine derivatives, hCMV-inhibitor complexes formed with thienoxazinone **3** (MW 392), the nitroso derivative **16** (MW 390) and the thienopyrimidinone **19** (MW 391), were investigated by mass spectrometry.[12] Incubation of **3** for 2 min with CMV protease at a 1:1 molar ratio resulted in the formation of a covalent complex whose average molecular mass (+/- 3 Da) was 390 Da higher than that of hCMV[13], consistent with the addition of a single molecule of **3** (or the nitroso derivative) to the enzyme. After 1 h the complex accounted for approximately 55% of total protein content of the sample and was subsequently digested with trypsin. Analysis of the tryptic peptides and comparison with those obtained from a control digest indicated that Ser^{132} within the peptide fragment (GPVSP LQPDK VVEFL SGSYA GL**S**^{132}LS SR) had been modified. This is believed to be an acyl-enzyme adduct formed through opening of the oxazinone ring by Ser^{132} and has a MW increase of **[3]**-16 Da compared with the control fragment, consistent with formal addition of the aniline **7**. Although not observed at the initial time point, nor at 1 h, transformation to the aniline must have occurred under the conditions (notionally non-reductive) of the digest (typical digest time 3 h).

Unlike **3**, no covalent adduct was observed for the thienopyrimidinone **19** at the initial time point but an adduct was observed after 1 h with a molecular mass consistent with addition of a single molecule of **19** to the protease. Tryptic digestion of the sample and subsequent LC/MS analysis indicated that the binding site was within a 20 amino acid peptide (residues 137-156, R**C**^{138}DDV EVATS LSGSE TTPFK) that did not contain the active site serine. MS/MS analysis of the doubly charged ion (1266.5 m/z) suggested that the ligand had reacted formally via nitroso addition to Cys^{138} to form the sulfinanilide[10,11] **24**. MALDI MS indicated a monoisotopic molecular mass of 2529.6 Da for the peptide fragment consistent with formal oxidation of the hydroxylamine to the nitroso analogue (theoretical 2530.0 Da).



24



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Binding of the nitroso derivative **16** was more complicated. After 15 minutes incubation of a 1:1 molar ratio of **16** and hCMV two E:I complexes were observed with molecular masses consistent with the addition of either 1 or 2 molecules of inhibitor to the enzyme and accounting for ~ 40% and 50% of total protein content respectively. During the course of incubation the concentration of the +2 I complex decreased, consistent with

decomplexation of one of the binding sites. Subsequent digestion and LC/MS/MS analysis of the more stable +1 I complex indicated that the 137-156 tryptic peptide fragment had been modified. The MS/MS spectrum, obtained from the doubly charged ion (1313 m/z), of this peptide contained the same major fragment ions as those observed in the peptide modified by **19,** indicating binding to Cys^{138}, but with a 92 Da mass increase compared with **16**. This was assigned as the glycerolysis turnover product **25**, resulting from the high concentration of glycerol in the storage buffer. The same modified peptide was also observed, although at a much reduced intensity, on extended incubation of **3** with hCMV protease.

To confirm the primary binding site of **3** and **16** glycerol was removed by dialysis against the assay buffer. The incubations were repeated but after 5 min excess ligand was removed, the complexes analysed by LC/MS, digested and the tryptic peptides analysed by LC/MS/MS. Under these conditions both compounds yielded complexes with molecular masses (± 3 Da) of 390 and 391 Da higher than hCMV which is consistent with the MW of the hydroxylamine and nitroso adducts. LC/MS/MS of the tryptic peptides indicated attachment to Ser^{132} with a mass increase of 376 Da consistent with binding of the aniline **7**.

In summary, in the absence of glycerol, MS data establish the primary binding site for the 1:1 complexes of **3** and **16** with hCMV protease as Ser^{132}. The fact that the Cys^{138}-containing tryptic peptide fragment (albeit at reduced intensity) was observed in the studies using **3** and glycerol-containing media is evidence that subsequent/parallel reaction via a nitroso derivative cannot be ruled out. At no time during the studies with **3** and **16** was any peptide observed which linked Ser^{132} and Cys^{138} via the ligand. With the thienopyrimidinone **19**, as expected, no adduct formation was observed at Ser^{132}, only the sufinanilide adduct of Cys^{138} being detected. The lack of an immediate adduct for this hydroxylamine, suggests inhibition via oxidation to the corresponding nitroso derivative which then undergoes attack by Cys^{138}. Intermediates such as thionitroxide radicals,[10] ArN(O·)SR, and semimercaptals[11], ArN(OH)SR, have been postulated to rationalise the formation of sulfinanilides, hydroxylamines and anilines, together with disulfides in the reaction of nitroso derivatives with thiols. In the study reported here, however, there was no evidence of disulfide bridging in the fragment peptides of the digest.

Cys^{161} (which lies in the prime side binding groove) can participate in additional interactions with a thienoxazinone inhibitor possessing an appropriately positioned Michael acceptor.[8c] Baum *et al.*[14] have demonstrated that flavins induce inhibition of hCMV protease by intramolecular Cys^{138}-Cys^{161} disufide bond formation. Flynn *et al.*[15] have also reported the inhibition of hCMV with a series of benzimidazolylmethyl sulfoxides which appear to involve oxidative modification of cysteine residues, although in this case it is not clear which particular residue is involved. In none of the experiments described in the current work, however, was involvement of Cys^{161} detected, although its temporary participation cannot be discounted.

In conclusion, although the reasons for the increased potency of the thienoxazinone derived hydroxylamine and nitroso compounds have not been definitively established, the compounds nevertheless

remain some of the most potent, non-peptide inhibitors of CMV protease prepared to date. The novel inhibitory mechanism of hCMV utilising Cys^{138} complements those previous publications describing inhibition of the enzyme through Cys^{161} and Cys^{138}-Cys^{161} bridging.

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