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2-Chloro-3-Substituted-1,4-Naphthoquinone Inactivators of Human Cytomegalovirus Protease

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Abstract: A random screening approach has identified 2-chloro-3-substituted-1,4-naphthoquinones as potent inactivators of HCMV protease. Enzyme inactivation is due to modification of Cys202. Two of the most potent compounds maintain activity against HCMV in a plaque reduction assay.

Human cytomegalovirus (HCMV) disease is the most common life-threatening opportunistic viral infection in the immunocompromised. It is the primary cause of death in recipients of bone marrow and renal transplants[1,2] and is the most prevalent serious infection in AIDS patients, frequently giving rise to pneumonitis and retinitis.[3,4] HCMV protease plays a critical role in capsid assembly and viral maturation[5,6] and is an attractive target for antiviral chemotherapy. HCMV protease is a serine protease, the crystal structure of which has recently been reported, revealing a novel protein fold and novel catalytic machinery involving Ser-132 and two histidine residues (His-63, His-157).[7-10] Strategies to inhibit HCMV protease include the use of peptidomimetics,[11,12] β-lactams[13-15] and sulfhydryl-modifying molecules.[16-18] Herein we wish to report the discovery and mode of action of 2-chloro-3-substituted-1,4-naphthoquinone inhibitors of human cytomegalovirus protease.

A random screening approach using an SPA assay[19] identified 2-chloro-3-(2,6-dioxo-4,4-dimethylcyclohexyl)-1,4-naphthoquinone (**1**) as a moderate enzyme inhibitor (IC_{50}=150 μM). Further screening of around 100 analogues afforded a number of very potent compounds capable of rapid and irreversible inactivation of the proteolytic enzyme. A selection of those tested is shown in Table 1.[20] The most potent compounds were also tested in an HPLC assay.[21] This confirmed the potent activities and approximate rank order of the naphthoquinone derived inhibitors against HCMV protease.

It is notable that the reactivity of the 2-chloro-3-substituted naphthoquinone derivatives as Michael acceptors correlates with the level of protease inhibition. Thus, the most reactive systems, especially those with β-keto ester and β-diketone substituents at the 3-position, are the most active protease inhibitors. HCMV protease contains 5 cysteine residues. Protease inhibition by covalent modification of Cys84, Cys87, Cys138 and Cys161 has been reported,[16-18] as has protease inactivation by intramolecular disulfide bond formation between Cys84-Cys87 and Cys138-Cys161.[16,17]

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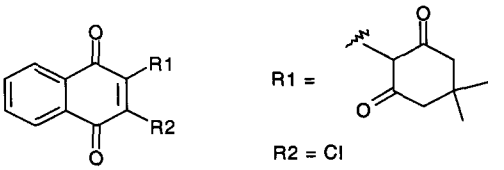
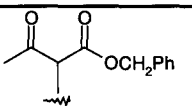
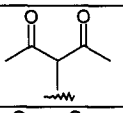
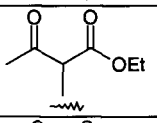
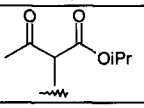
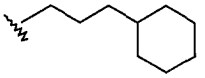
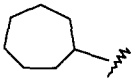
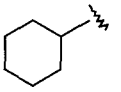
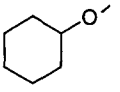
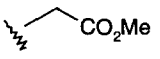
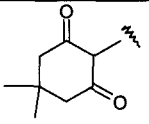


1 IC_{50} = 150 μM

Table 1 : Activity of selected 2,3-disubstituted-1,4-naphthoquinones against HCMV protease

Cpd No.	R1	R2	IC_{50} (μM) SPA assay	IC_{50} (μM) HPLC assay
2		Cl	0.73	0.70
3		Cl	0.8	0.72
4		Cl	1.45	4.8
5		Cl	2.68	9.1
6	Cl	Cl	3.9	5.3
7		Cl	12.35	NT
8		Cl	67	NT
9			110	NT
10		Cl	148	NT
11		OH	11% Inhib at 150 μM	NT

To investigate the interaction of these compounds with HCMV protease, the protease was incubated with an equimolar amount of the 1,3-diketone derivative **3**, and analysed by electrospray mass spectrometry (ESMS). A modified protein of molecular weight 254 Da higher than the native

protein was observed, consistent with an addition-elimination process, in which reaction of **3** with the protein is followed by the loss of HCl. Tryptic digest and sequencing by MS/MS identified the modified amino acid to be Cys202, the first example of protease inactivation via modification of this Cys residue (Figure 1).

The compounds were examined for their anti-HCMV activities in a plaque assay (strain AD169 in MRC-5 cells).[22] Most of the compounds had a very narrow therapeutic window, with cell toxicities close to the IC_{50} values for viral inhibition. However, the β-keto ester derivatives **4** and **5** had IC_{50} values of 10 μM and 13 μM respectively, with no observed toxicity up to 100 μM in MRC-5 cells.

As the molecules inactivate a surface cysteine residue, we investigated the issue of selectivity by testing compounds **1-5** against the prototypical serine proteases thrombin and human neutraphil elastase (HNE) at 100 μM. No inhibition was observed against thrombin, whilst only compound **2** had moderate inhibition against HNE (IC_{50} = 30 μM). We also assessed their stability towards glutathione as a typical thiol nucleophile, and found that they did react covalently with glutathione in DMSO at ambient temperature over several hours.

In summary, we have identified potent irreversible naphthoquinone inhibitors of HCMV protease which covalently modify Cys202. Certain examples retain activity in a viral plaque assay, are selective over thrombin and HNE, but have limited chemical stability towards biologically relevant nucleophiles. The design of inhibitors with reduced reactivity and specific enzyme recognition at a site away from the active site would be a formidable challenge.

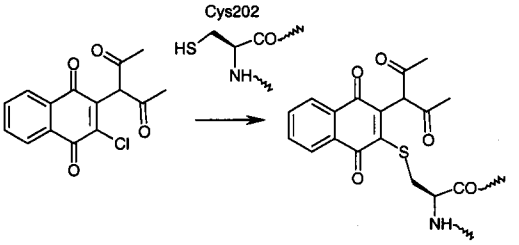


Figure 1. Proposed mechanism of enzyme inactivation

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19. SPA Assay description: HCMV protease (AD169) was cloned with a single mutation (A143N) to reduce internal cleavage, expressed in *E.coli*, and highly purified by ion exchange and gel filtration chromatography. Appropriate dilutions of compound were pre-incubated for 15 mins. with 3.6 μM protease in 15 μl reaction volumes, containing 30% glycerol and 0.1M Tris-HCl pH7.7, before addition of 15 μl of similar buffer containing 8.9 nM substrate (Biotin-Aha-RGVVNASSRL(^{3}H)G, Amersham, 125Ci/mmol, 1mCi/ml). Reactions were incubated at 25 °C for 60 mins, and stopped by addition of 170 μl of 3mM zinc chloride containing 40 μg of streptavidin-SPA beads (Amersham NK8972). Inhibition of enzyme activity was calculated by comparison to controls.
20. Compounds were prepared according to established procedures. See Boehm, P.; Cooper, K.; Hudson, A.T.; Elphick, J.P.; McHardy, N. *J. Med. Chem.* **1981**, 24, 295. Hudson, A.T.; Pether, M.J.; Randall, A.W.; Fry, M.; Latter, V.S.; McHardy, N. *Eur. J. Med. Chem. Chim. Ther.* **1986**, 21, 271.
21. HPLC assay description: HPLC assays were essentially similar to SPA assays[19] except that, after pre-incubation , substrate (peptide RGVVNASSRLAK) was added to a final concentration of 100 μM, and reactions were stopped after 60 mins by addition of 30 μl of 0.2% TFA in water. Samples were filtered and examined by HPLC on a C18 reverse phase column (ABI Bronlee (10x4.6) No. 0711 0021). Substrate and product peaks were determined and used to calculate the level of cleavage.
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