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INHIBITION OF HUMAN CYTOMEGALOVIRUS PROTEASE BY BENZOXAZINONES AND EVIDENCE OF ANTIVIRAL ACTIVITY IN CELL CULTURE

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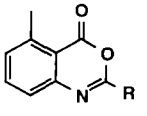
Abstract: A series of 2-amino substituted benzoxazinones were prepared that potently inhibit human cytomegalovirus protease in vitro. Selected compounds demonstrate antiviral activity in cell culture, selectivity relative to chymotrypsin and elastase and stability with respect to hydrolysis in human plasma.

Human cytomegalovirus (hCMV), a member of the Herpesviridae family, infects a large portion of the population[1] and causes morbidity and mortality, particularly in immunocompromised individuals.[2] Current therapies depend on inhibition of viral DNA polymerase using nucleoside analogs such as gancyclovir and foscarnet. In addition to some dose limiting side effects,[3] these compounds are hampered in their ability to penetrate the blood brain barrier to effectively treat the CNS manifestations of the infection. Herpesviruses, including hCMV, encode a serine protease responsible for the processing of capsid assembly protein leading to the subsequent packaging of DNA into the mature capsid.[4] A critical function of the protease is to cleave the assembly protein between two separate, conserved alanine—serine sequences. Inhibition of this protease cleavage is considered to be an attractive approach for drugs targeted to treat hCMV infection since mutations in the protease gene of a related herpesvirus, HSV-1, are deleterious to viral replication.[5]

2-Substituted benzoxazinones[6] **1** have been well characterized as inhibitors of a variety of mammalian serine proteases through an alternate-substrate mechanism involving a stable acyl—enzyme complex. In particular, such compounds have demonstrated significant potency against human leukocyte elastase (HLE). Krantz[7] and coworkers have reported that 2-amino-5-methyl substituted benzoxazinones possess requisite HLE inhibitory potency and intrinsic stability in aqueous buffer. Furthermore, compounds of this subclass (**2** and **3**) selectively inhibit HLE and exhibit significant stability in human plasma.[8] Based on these findings we targeted a subclass of benzoxazinones that would possess potency and selectivity for the viral protease versus mammalian proteases, retain plasma stability, and exhibit antiviral activity in cell culture. Recently, the inhibition of a related herpesvirus protease, HSV-1 protease, by a class of benzoxazinones has been reported.[9] In a consecutive paper, inhibition of hCMV protease by a unique class of mechanism-based inhibitors has also been reported.[10]



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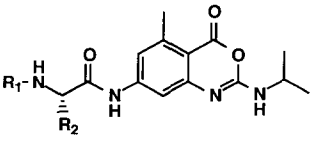
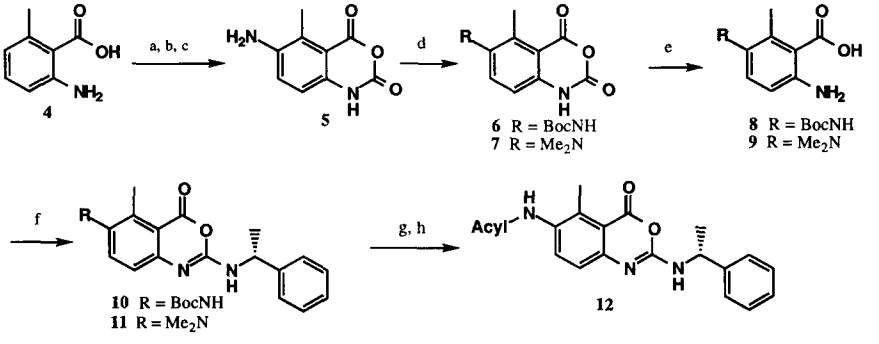


2 R_1 = 4-Cl-phSO$_2$ R_2 = $(CH_2)_2CO_2H$
3 R_1 = 4-Cl-phSO$_2$ R_2 = $(CH_2)_4NH_2$

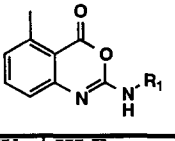
The compounds were prepared according to the general method outlined in Scheme 1. Condensation of anthranilic acid **4** with carbonyldiimidazole followed by regioselective nitration and subsequent catalytic hydrogenation afforded the 6-amino substituted isatoic anhydride analog **5**. Compound **5** could be used in one of two ways. Either protection with boc anhydride or reductive alkylation using aq formaldehyde afforded the compounds **6** and **7**, respectively. Alkaline hydrolysis and adjustment of the pH afforded the appropriately substituted anthranilic acids, **8** and **9**. These compounds were converted to benzoxazinones **10** and **11** in a one-pot procedure involving condensation with an isocyanate followed by dehydrative cyclization using water-soluble carbodiimide (WSCI). Further analogs of **10** were prepared by removal of the boc group and acylation with a variety of acid chlorides.



Scheme 1: General method for the preparation of substituted benzoxazinones.
a. carbonyldiimidazole, dioxane; b. HNO_3, H_2SO_4; c. H_2 5% Pd/C. THF; d. Boc_2O, cat. DMAP, DMF or 37% aq formaldehyde, 5% Pd/C, THF; e. 1 N NaOH then adjust to pH 4; f. (*R*)-(+)-α-methylbenzyl isocyanate, Et_3N, DMF then WSCI; g. HCl (g), EtOAc; h. "acid chloride", Et_3N, DMF.

Compounds **13—16** were prepared directly from **4** through condensation with the appropriate isocyanate and cyclization as described above.

Table 1: Enzyme Inhibition and Antiviral Activity



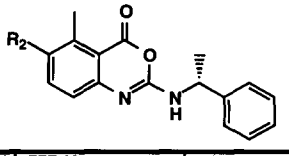
cpd.	R_1	hCMV	HLE IC_{50} (μM)	chymo	antiviral activity EC_{50}/TC_{50} (μM)
13	1-Pr	4.0	0.033	2.0	- -
14	(1*R*)-1-phenylethyl	0.92	0.092	0.088	23/>100
15	(1*S*)-1-phenylethyl	1.6	0.044	0.18	63/>100
16	(1*R*)-(4-bromo-phenyl)ethyl	0.46	0.020	0.14	- -

Enzyme inhibitory activity was measured in a colorimetric assay using the appropriate *p*-nitroanilide substrates.[11] Antiviral activity was determined using an hCMV variant expressing a beta-galactosidase reporter gene.[12] The known compound[7] **13** (Table 1) did in fact inhibit hCMV protease as well

as HLE and chymotrypsin, as expected. Compound **14** was somewhat more potent and became an important lead compound since it also possessed antiviral activity in cell culture. The enantiomer **15** and *p*-bromo analog **16** were also active but appeared to offer no advantage over the lead compound **14**.

Further optimization of **14** in an effort to improve enzyme selectivity led to a series of 6-substituted benzoxazinones. The 6-dimethylamino analog **17** (Table 2) afforded excellent potency and a significant shift in selectivity away from HLE but without affecting the chymotrypsin selectivity. Acylation of the 6-amino group led to compounds with weakened activity for mammalian proteases. For example, the *t*-butylcarbamate analog **18** showed good enzyme selectivity. The *p*-methoxybenzenesulfonamide **19** and the nicotinamide **20** displayed enhanced potency with **20** being more selective with respect to the other serine proteases. Compound **20** was insoluble under the assay conditions at 100 μM. Introduction of a basic functional group in the form of a dimethylglycine amide or pyrrolidineacetamide, **21** and **22**, led to compounds with even greater selectivity. In particular, **22** showed little activity towards HLE and chymotrypsin at 100 μM while retaining activity towards the viral protease.

Table 2: Enzyme Inhibition and Antiviral Activity



compd	R_2	hCMV	HLE IC_{50}(μM)	chymo	antiviral activity EC_{50}/TC_{50} (μM)
17	Me_2N	0.24	3.2	0.065	31/>100
18	BocNH	1.3	40	42	10/62
19		0.27	66% @ 100 μM	3.6	8.5/55
20		0.72	29% @ 10 μM	28% @ 10 μM	20/>100
21		2.2	67% @ 100 μM	2.6	46/>100
22		2.4	36% @ 100 μM	47% @ 100 μM	30/70

These compounds were also evaluated for their ability to prevent viral replication in the rapid cell culture assay. Compared to compound **14**, compounds **17**, **20**, and **21**, showed comparable antiviral activity. However, compounds **18**, **19**, and **22** displayed some toxicity in the mock infected cell culture.

Table 3: *In vitro* half-life in human plasma.

compd	IC_{50} (μM)	$T_{1/2}$ (min)	compd	IC_{50} (μM)	$T_{1/2}$ (min)
13	4.0	107	**18**	1.3	30
14	0.92	67	**19**	0.27	50
15	1.6	70	**20**	0.72	31
16	0.46	50	**21**	2.2	32
17	0.22	127	**22**	2.4	32

In vitro human plasma stability[13] was determined by monitoring the loss of fluorescence emission of the parent benzoxazinone ring system. The half-lives are shown in Table 3. These compounds displayed $T_{1/2}$'s

from 22 to 127 minutes and don't appear to correlate with relative enzyme potency. In fact, one of the more potent compounds, **17**, possessed the longest half-life. Increased half-life could be a function of the increased stability of the oxazinone ring as a result of increased electron density provided by the dimethylamino residue. Alternatively, **17** may be less susceptible to degradation by the host of esterases and amidases present in plasma.

In conclusion, a series of 6 substituted 2-aminobenzoxazinone analogs has been prepared that show potent activity towards the target enzyme and demonstrate antiviral activity in cell culture. Modification of the substituent at the 6 position modulates both enzyme selectivity and plasma stability without adversely affecting potency towards the viral protease.

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11. The hCMV protease assay employed succinyl-ala-gly-val-val-asn-ala-*p*-nitroanilide as substrate. Inhibitor (10 µL in DMSO) and enzyme (100 µL, 4.8 µg/mL in assay buffer [10 mM Na phosphate, 150 mM Na acetate buffer, pH 7.4, with 0.1% CHAPS and 20% glycerol]) were incubated in a 96-well assay plate (Falcon 3910) for 30 min at room temperature. Substrate (50 µL of 9 parts assay buffer and 1 part 20 mM substrate in DMSO) was then added and the absorbance at 405 nm monitored in an absorbance reader. Activities were expressed as absorbance change per minute and compared to controls lacking inhibitor.
12. Hippenmeyer, P. J.; Dilworth, V. M. *Antiviral Res.* **1996**, *32*, 35. The TC_{50} is the concentration of compound that decreases cell number 50% in a matched, mock-infected plate as measured by crystal violet staining. TC_{50}'s greater than 100 µM were unatainable due to solubility limitations of the test compounds and the amount of DMSO that could be added to the assay. The EC_{50}/TC_{50} for gancyclovir in this assay is 8.9/>100 uM. Adler, J.; Mitten, M.; Norbeck, D.; Marsh, K.; Kern, E. R.; Clement, J. *Antiviral Res.* **1994**, *23*, 93.
13. Stability of benzoxazinones in human plasma was determined by monitoring the decrease in fluorescence upon hydrolysis of the oxazinone ring. Single donor human plasma (196 µL) was added to 4 µL of 1 mM benzoxazinone in DMSO in a 96-well assay plate (Dynatech Microlite 1) and the fluorescence (excitation: 365 nm broadband filter, emission: 450 nm narrowband interference filter and 400 nm UV blocking filter) monitored in a fluorescence reader. Fluorescence measurements were corrected for background using controls lacking inhibitor, and the resulting values plotted vs. time. The data were fit to a single exponential decay function, $f_t = f_0 * \mathrm{Exp}(-kt)$, where f_0 and f_t are the fluorescence at time zero and at time t, resectively, and k is the decay constant. Half-life was determined as the average of at least 3 separate determinations (three different plasma donors), by calculating half-life = ln(2)/k.

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