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BENZOTHIOPYRAN-4-ONE BASED REVERSIBLE INHIBITORS OF THE HUMAN CYTOMEGALOVIRUS (HCMV) PROTEASE

Dashyant Dhanak,*[a] Richard M. Keenan,[a] George Burton,[a] Arun Kaura,[a] Michael G. Darcy,[a] Dinubhai H. Shah,[a] Lance H. Ridgers,[a] Ann Breen,[a] Paddy Lavery,[b] David G.Tew,[b] and Andrew West[b]

[a]*SmithKline Beecham Pharmaceuticals, 1250 South Collegeville Road, P.O. Box 5089, Collegeville, PA 19426-0989, U.S.A.*

[b]*SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, The Pinnacles, Harlow, Essex, CM19 5AG, U.K.*

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Abstract: A novel class of CMV protease inhibitors based on a benzothiopyran-*S,S*-dioxide nucleus has been discovered. Enzyme kinetic data supports a reversible mode of inhibition for a representative member of this class, 2-(3-pyridyl-N-oxide)benzothiopyran-4-one-*S,S*-dioxide, **1**. Experiments in the presence and absence of the disulfide reducing agent DTT suggest that the inhibition by **1** is not due to oxidative inactivation of the enzyme. Also presented are results of some SAR studies of the benzothiopyranone ring system.

Cytomegalovirus (CMV) is a member of the β-herpes virus family and is the cause of significant disease manifestations, particularly in immunocompromised individuals such as AIDS patients and those undergoing transplant surgery.[1] Although a number of therapies are currently available for the treatment of CMV infections, all have serious drawbacks including toxicity and only limited effectiveness.[2]

The UL80 gene of CMV encodes a serine protease that is expected to be essential for viral replication in cell culture based on homology with the essential HSV protease.[3] It is anticipated that potent inhibitors of this protease will ultimately prove to be effective antiviral agents in the clinic. A number of reports have appeared recently disclosing low molecular weight inhibitors of this protease activity in vitro, and some have also shown antiviral activity in CMV-infected human cell lines. These inhibitors contain classical serine protease inhibitor motifs based on an activated carbonyl group such as oxazinones[4a] and substrate-based peptidyl α-ketoamides,[4b,4c] as well as mechanism-based inhibitors such as spirocyclopropyl-oxazolones, benzylidine N-sulfonyloxy imidazolones,[4d] and β–lactams.[4e,4f] We report herein the discovery of a novel class of CMV protease inhibitors incorporating the benzothiopyran-4-one nucleus and present kinetic data supporting a reversible mode of inhibition for a representative member of the series.

High-throughput screening identified 2-(3-pyridyl-N-oxide)benzothiopyran-4-one-*S,S*-dioxide **1** as a potent inhibitor of CMV protease (IC_{50} = 0.6 μM, Table 2). Since **1** contains Michael acceptors in the form of both an α,β-unsaturated enone and an α,β-unsaturated sulfone, and since irreversible inhibition of various serine and cysteine proteases with such systems has been reported,[5] we were interested in studying the reversibility of the interaction of **1** with hCMV protease. We had previously found that active site acylators are able to alter the

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HPLC retention time of CMV protease (Tew and Ashman, unpublished results). However, incubation of an excess of **1** with CMV protease for 30 min followed by reverse-phase HPLC analysis of the enzyme showed no difference in the retention time of the protease and protease + **1**, suggestive of the lack of a covalently bound enzyme/inhibitor complex. In addition, electrospray ion/mass spectrometry (ES/MS) of the enzyme after 30 min incubation with an excess of **1** showed only the presence of native protease of molecular weight 28175 daltons (Figure 1).[6] Also, incubation of a mixture of an excess of **1** and protease for 15 min followed by dilution with substrate such that [substrate] >> [inhibitor] led to recovery of enzyme activity, again indicative of a reversible enzyme/inhibitor interaction (data not shown).

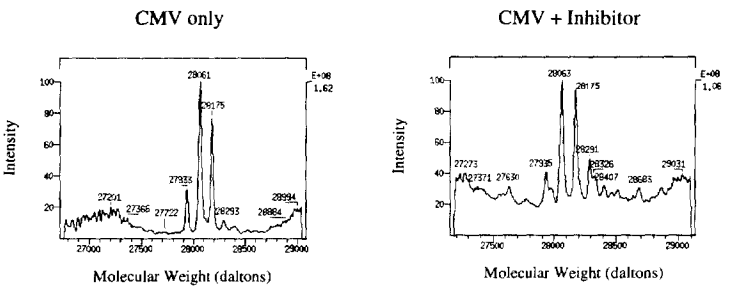


Figure 1. ES/MS of hCMV protease ± inhibitor **1**

The sensitivity of CMV protease to inactivation via oxidative intramolecular disulfide formation between Cys138 and Cys161 has been reported.[7] Both residues are in or near the active site in the crystal structure of the protease[8] and presumably are able to interact both with each other and substrate or competitive inhibitors. Furthermore, the pyridine N-oxide moiety or sulfone in **1** has the potential to undergo reduction with concomitant oxidation of the enzyme. To determine whether the inhibition due to **1** was mediated via oxidation of the protease to the inactive disulfide, a tenfold excess of **1** was incubated with the protease for 15 min and the resulting enzyme:inhibitor (E:I) complex gelfiltered to remove excess inhibitor. The eluate containing CMV protease was then assayed for activity in the presence or absence of the disulfide reducing agent dithiothreitol (DTT). The results expressed as a percentage of control (no exposure to inhibitor) are shown in Table 1. No significant difference between the protease activity in samples with or without DTT was observed indicating that little, if any, oxidative inactivation had occurred on exposure of protease to **1**. Taken together, these data provide strong evidence that **1** interacts in a reversible manner with CMV protease and the observed inhibition is not the result of an oxidative modification of the enzyme.

Table 1. Results of DTT experiment to determine possible inhibition via oxidative disulfide bond formation

	Average* Activity	
Sample	**+ 100 μM DTT**	**No DTT**
Protease/inhibitor/DMSO/buffer	76%	71%
Protease/DMSO/buffer	79%	81%

* mean of two determinations

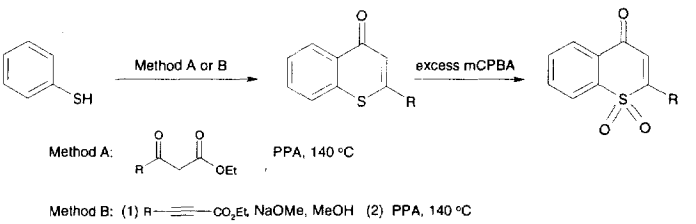
In order to assess the selectivity of the benzothiopyranone inhibitors towards other proteolytic enzymes, **13** was assayed for the inhibition of some serine and cysteine proteases and also of a serine lipase[9]. In all cases **13** was found to be, at best, only weakly active (IC_{50} > 10 μM) indicating a high degree of selectivity for hCMV protease. In addition, the propensity of **13** towards non-specific nucleophilic addition was also determined by incubation with an excess of propyl mercaptan in d^6-DMSO solution with the reaction being monitored by ^1H

NMR. No significant incorporation of a thiopropyl group by Michael addition was evident after 18 h, confirming the relative stability of **13** under these conditions.

Synthesis

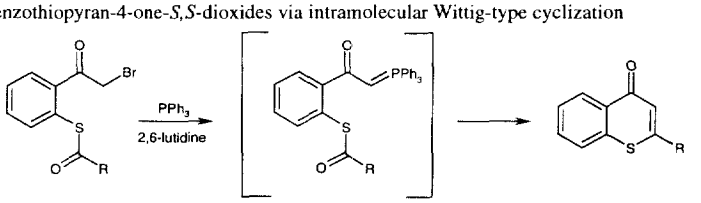
Analogs of **1** were prepared in general by one of two methods. The acid-mediated cyclization of thiophenol and either a β-keto ester[10] or a propiolate[11] was used to prepare the benzothiopyranone nucleus in compounds **1–16** and **19–20** (Scheme 1).[12]

Scheme 1. Synthesis of benzothiopyran-4-one-*S,S*-dioxides via acid-mediated cyclization



Alternatively, an intramolecular Wittig-type cyclization of the phosphorane derived from a 2-(acylmercapto)phenacyl bromide[13] was used to prepare the thiopyranones **17–18** (Scheme 2).

Scheme 2. Synthesis of benzothiopyran-4-one-*S,S*-dioxides via intramolecular Wittig-type cyclization

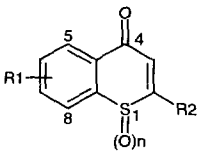


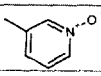
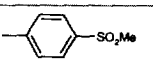
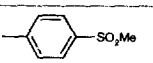
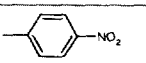
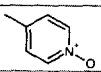
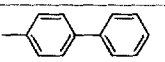
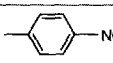
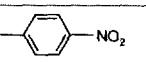
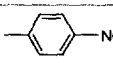
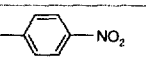
Oxidation of the benzothiopyranone using stoichiometric amounts of mCPBA gave a mixture of the sulfoxide and sulfone which were separable by chromatography; use of an excess of oxidant cleanly furnished the sulfone. Similarly, the pyridyl N-oxides **1** and **4** were prepared by oxidation of the appropriate benzothiopyranone with excess mCPBA. Selective reduction of the N-oxide to the pyridine was achieved in good yield using PCl_3.[14] The biological activity of all compounds was evaluated by following the increase in fluorescence resulting from the cleavage of a quenched fluorescent peptide substrate.[15]

Results and Discussion

The oxidation level of the sulfur had a marked effect on potency (Table 2) with optimal activity being associated with the sulfones. The corresponding sulfides were inactive and the racemic sulfoxides showed only weak activity. Removal of the N-oxide in either **1** or the 4-pyridyl isomer **4** to the pyridines (**3** and **5**) gave a moderate increase in potency whereas replacement of the heterocycle with a phenyl ring (**8**) led to a dimunition of activity. However, activity could be restored to the 2-phenyl series by the introduction of electron withdrawing groups - both the 4-methylsulfone **12** and the 4-nitro derivative **13** were ~20-fold more potent than the parent compound **8**. The 2-thienyl system in **14** served as an effective replacement for the pyridine ring in **1** but offered no advantage in terms of activity. In contrast to both **1** and **14**, the 3-thienyl isomer **15** showed no inhibitory activity against hCMV protease.

Table 2. IC_{50} values for benzothiopyran-based CMV protease inhibitors



Compound	R1	R2	n	IC_{50} (μM)*	Compound	R1	R2	n	IC_{50} (μM)*
1	H		2	0.6	11	H		0	NI
2	H		0	NI	12	H		2	0.1
3	H		2	0.2	13	H		2	0.06
4	H		2	0.6	14	H		2	0.46
5	H		2	0.1	15	H		2	NI
6	H		0	NI	16	H		2	0.06
7	H		1	10	17	H		2	1.4
8	H		2	1.5	18	H		2	NI
9	5-Me		2	0.1	19	8-Me		2	0.11
10	6-CO_2Et		2	0.18	20	8-iPr		2	0.18

*NI = noninhibitory defined as $IC_{50} > 34$ uM

The potency enhancing effect obtained with an electron-deficient 2-aryl substituent could also be mimicked by lipophilic groups, as illustrated by the biphenyl derivative **16**, which was equipotent to **13**. However, lipophilicity alone was not sufficient to obtain good activity since both the 2-octyl and the 2-tert-butyl analogs (**17** and **18**, respectively) were only marginally active. In an attempt to improve potency further, a variety of substituents were introduced into the benzo ring of the benzothiopyran nucleus. However, these substituents did not markedly influence the activity, suggesting that this part of the molecule was not involved in making extensive interactions with the enzyme.

Summary

Benzothiopyran-*S,S*-dioxides have been found to be potent inhibitors of the hCMV protease in vitro. Enzyme kinetic data obtained with **1**, a prototypical member of this class of inhibitor, supports a reversible mode of

inhibition that does not involve oxidative inactivation of the enzyme. SAR studies around the 2-position indicate that electron-deficient aryl rings or extended π-conjugates at this position can confer good protease inhibitory activity. Further details on the investigation into the mechanism of action and cell-based activity of these compounds will be reported in due course.

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15. **Assay conditions:** Stock solutions of inhibitors were made up and serially diluted in 100% DMSO and activity measurements were carried out in opaque 96-well reaction plates. Briefly, inhibitor was diluted into buffered protease (final concentration ~25nM) to give a concentration range spanning 34 - 0.33μM. The assay mixture (200 μL total volume containing 50 mM HEPES/150 mM NaCl/1 mM EDTA/0.01% PEG 3,400/0 in 30% sucrose, pH 7.5) was incubated at 27 °C for 15 min before the addition of peptide substrate to give a final substrate concentration of 10μM. The substrate peptide used was Dabs-RGVVNASSALAKK-DansII (where Dabs = 4-[4- (dimethylamino)phenylazo]benzenesulfonyl and DansII = 6-(dimethylamino)naphthalene-1-sulfonyl). The plate was measured for 15 min at 27 °C with a SLT Fluostar fluorometric plate reader, Ex 355nm/Em 496nm. The IC_{50} was determined from the measured fluorescence change using Grafit[16] software.

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