Structure and Design of Potent and Selective Cathepsin K Inhibitors

Dennis S. Yamashita,[†] Ward W. Smith,[‡] Baoguang Zhao,[‡] Cheryl A. Janson,[‡] Thaddeus A. Tomaszek,[§] Mary J. Bossard,[§] Mark A. Levy,[§] Hye-Ja Oh,[†] Thomas J. Carr,[†] Scott K. Thompson,[†] Carl F. Ijames,[||] Steven A. Carr,[||] Michael McQueney,[⊥] Karla J. D'Alessio,[⊥] Bernard Y. Amegadzie,[▽] Charles R. Hanning,[▽] Sherin Abdel-Meguid,[‡] Renee L. DesJarlais,[||] John G. Gleason,[†] and Daniel F. Veber*,[†]

Departments of Medicinal Chemistry, Macromolecular Sciences, Molecular Recognition, Structural and Physical Chemistry, Protein Biochemistry, and Gene Expression Sciences SmithKline Beecham Pharmaceuticals, 709 Swedeland Rd. King of Prussia, Pennsylvania 19406

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Cathepsin K, a cysteine protease of the papain superfamily, is selectively expressed in osteoclasts and has been implicated in the process of bone resorption. Therefore, inhibition of Cathepsin K offers a promising mechanism for the treatment of diseases characterized by excessive bone loss such as osteoporosis.[1] We report the successful design of selective, "quiescent", reversible, inhibitors of Cathepsin K based on a poorly electrophilic 1,3-bis(acylamino)-2-propanone scaffold, an approach inspired by the overlay of aldehyde-type structures bound in different modes elucidated by X-ray cocrystallography on papain. The design hypothesis has been confirmed by X-ray crystallography of inhibitors bound to Cathepsin K. The wide range of therapeutic potential postulated for inhibitors of cysteine proteases in the areas of arthritis, cancer, viral, and parasitic disease suggests targets for further application of this approach.[2]

In order to minimize potential immunological complications in drugs given chronically, a key constraint of protease inhibitor design has been to avoid the presence of intrinsically reactive groups that might derivatize the side chain or backbone elements of proteins. Notable therapeutic successes toward this end have been seen with inhibitors of the metalloprotease, angiotensin converting enzyme, and inhibitors of the aspartyl protease, HIV protease. No comparable examples exist for the serine and cysteine proteases for which inhibitors generally utilize reactive groups which covalently modify the enzyme for their efficacy. Krantz has discussed the "quiescent affinity" of acyloxy ketones for cysteine proteases,[3] and Rando has described β-lactams as mechanism-based inactivators of β-lactamases.[4] Palmer has reported time-dependent, nonselective inhibitors of Cathepsin K (and other cysteine proteases) based on a vinyl sulfone[5] first utilized by Hanzlik.[6] Nonetheless, such compounds ultimately become covalently and irreversibly linked such that the protein remains derivatized after denaturation and degradation, thereby offering the opportunity for undesired antigenic and immunogenic responses.[7]

Much structural information has been reported in the literature for members of the papain superfamily of cysteine proteases. Papain, the prototypical member, has yielded to an X-ray crystal structure with the peptide aldehyde inhibitor, leupeptin (**1**) bound,[8] providing detailed information relating to inhibitor binding sites which orient on the "unprimed"[9] side of the active site. An X-ray evaluation of a complex of papain with Cbz-Leu-Leu-Leu aldehyde (**2**), a close analog of leupeptin, revealed the binding orientation of this compound within the "primed" side of the active site, an unanticipated result since the S2′ pocket appears more poorly defined relative to the S2 hydrophobic pocket of papain. Binding only on one side of the catalytic cysteine by inhibitors such as leupeptin or **2**, thereby utilizing only half of the active site, may contribute to the relative lack of selectivity[10] of these inhibitors for the various members of the papain family. We generated an overlay structure between bound aldehyde **2** and leupeptin (**1**) to achieve hypothetical inhibitor **3** that spans the entire active site, which was further simplified (Figure 1). First, we deleted the side chains in our initial target. Second, on the basis of modeling, it appeared that Trp 184 (Cathepsin K numbering) would form a better aromatic−aromatic interaction with the phenyl ring of the Cbz group if **2** was shortened by one amino acid. This observation was consistent with our enzymatic studies, in that the dipeptide aldehyde, Cbz-Leu-Leu aldehyde, was a potent, time-dependent inhibitor of Cathepsin K ($k_{inact}/[I] = 3 \times 10^5$ M^{-1} s^{-1})).[11,12] Therefore, we chose the readily accessible symmetrical ketone **4** as our initial synthetic target. The reduced electrophilicity of a ketone[13] compared to an aldehyde made this compound all the more attractive.

Indeed, ketone **4**, although a relatively poor inhibitor of papain with a $K_{i,app} > 10$ μM, had a $K_{i,app}$ of 22 nM against our actual target, Cathepsin K (Figure 1). In addition to being a poor inhibitor of papain, it was selective against the other members of the papain family ($K_{i,app}$ Cathepsin L, 0.34 μM; Cathepsin B,[14] 1.3 μM; Cathepsin S,[15] 0.89 μM). Also, the $K_{i,app}$ of **4** was greater than 50 μM for the serine proteases trypsin and chymotrypsin.[16] We have been unable to detect any intrinsic reactivity of the ketone of **4** toward thiols such as (*p*-

[†] Department of Medicinal Chemistry.
[‡] Department of Macromolecular Sciences.
[§] Department of Molecular Recognition.
[||] Department of Structural and Physical Chemistry.
[⊥] Department of Protein Biochemistry.
[▽] Department of Gene Expression Sciences.

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(10) The $K_{i,apps}$ of leupeptin against Cathepsins B and S are 122 and 17 nM, respectively. Leupeptin was also determined to be a time-dependent inhibitor of Cathepsin L with a $k_{inact}/[I] = 1.5 \times 10^5$ M^{-1} s^{-1}.

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(12) Inhibitors were evaluated for inhibition against purified recombinant Cathepsin K as described in ref 1b.

(13) An example of a 1-(acylamino)-5-(alkylamino)-4-oxopentanoic acid inhibitor of the cysteine protease, interleukin-1β converting enzyme, has been reported: Mjalli, A. M. M.; Chapman, K. T.; MacCoss, M.; Thornberry, N. A.; Peterson, E. P. *Biorg. Med. Chem. Lett.* **1994**, *4*, 1965−1968.

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(16) Papain, trypsin, and chymotrypsin were purchased from the Sigma Chemical Company.

S0002-7863(97)02204-X CCC: $14.00

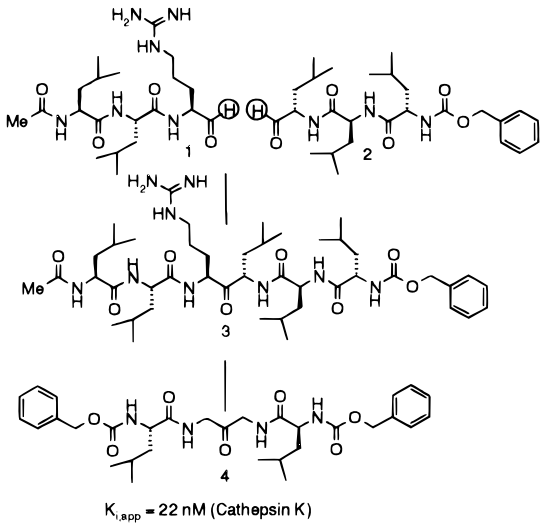
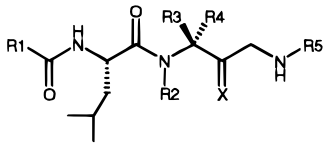


Figure 1. The evolution of 1,3-bis(acylamino)-2-propanone design.

Table 1. Activities of 1,3-Bis(acylamino)-2-propanone[18] vs Cathepsin K



	X	R1	R2	R3	R4	R5	$K_{i,app}$ (nM)
4	O	$PhCH_2O$	H	H	H	Cbz-Leu-	22
5	OH,H	$PhCH_2O$	H	H	H	Cbz-Leu-	>10000
6	O	$PhCH_2O$	Me	H	H	Cbz-Leu-	90
7	O	$PhCH_2O$	H	Me	H	Cbz-Leu-	36
8	O	$PhCH_2O$	H	H	Me	Cbz-Leu-	1200
9	O	$PhCH_2O$	H	H	H	4-Ph-O-PhCO-	67
10	O	$PhCH_2O$	H	H	H	4-Ph-O-$PhSO_2$-	1.8
11	O	4-pyridyl	H	H	H	4-Ph-O-$PhSO_2$-	13

methoxyphenyl)methanethiol in MeOD under neutral, basic (triethylamine), or acidic (AcOH) conditions. Consistent with our design that the cysteine of Cathepsin K would add to the ketone of **4**, structure activity data confirmed the importance of the ketone functionality for inhibition. The alcohol **5** (Table 1) is seen to lose nearly 3 orders of magnitude in potency in spite of retaining all of the peripheral binding elements of **4** other than the ketone.

Ketone **4** was shown to be a competitive inhibitor vs peptide substrate with Cathepsin K by a Lineweaver−Burk plot[17] and showed no evidence of time-dependent inhibition over the 30 min progress curve analysis. Rapid reversibility of inhibition was observed upon dilution using the protocol as described in ref 11 for determining dissociation rates (k_{off}) of reversible inhibitors bound to Cathepsin K. Furthermore, consistent with reversible inhibition, ketone **4** was subjected to incubation with Cathepsin K for 5 h, and the resulting protein was then analyzed by electrospray mass spectrometry showing no evidence of covalent modification of the enzyme. This is in contrast to Cbz-Leu-Leu-CH_2Br, a member of the classical α-halomethyl ketone cysteine protease inhibitors, in which the expected covalent adduct was observed (Cathepsin K + 374.8 Da) as a major product.[18]

The X-ray cocrystal structure of **4** bound to Cathepsin K was determined,[18] and the structure fits our original design model in that the data is consistent with (1) formation of a thiohemiketal between the ketone carbonyl and the active site Cys 25, (2) the inhibitor spans both the primed and unprimed sectors of the active site, (3) one of the side chains of the leucine groups fills the hydrophobic S2 pocket, and (4) aromatic−aromatic interactions between the two Cbz phenyl rings and the two aromatic residues Trp 184 and Tyr 67[19] are evident.

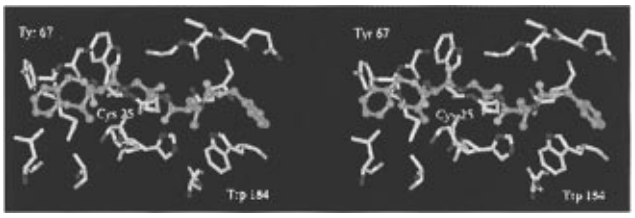


Figure 2. X-ray cocrystal structure of the active site of Cathepsin K and inhibitor **11**.

Because of the high potency and selectivity for Cathepsin K inhibition, we deemed **4** an excellent lead for further evaluation in a medicinal chemistry program. Our SAR investigation (Table 1) began by examining the effects of methylation. The N-methyl analog of **4**, ketone **6**, was 4-fold less active than **4**, and the activities of compounds with a methyl group at the α carbon of **4** were dependent on the absolute configuration. Ketone **7** derived from L-alanine had about the same potency as **4**; however, ketone **8**, which was derived from D-alanine, was 50-fold less potent than **4**.

Next, the peptidomimetic, 4-phenoxyphenyl group was designed to approximately span the distance of a Cbz amino acid (picking up the Trp 184 aromatic interaction) while eliminating the amino acid and the carbamate functionalities completely: amide **9** maintained good potency; whereas, the 4-phenoxyphenyl sulfonamide **10** was 10-fold more active than our original peptide-based lead. Good potency could be retained by replacing the remaining Cbz group with a water-solubilizing group such as the 4-pyridyl carbonyl in ketone **11**. Again, an X-ray cocrystal structure of **11** with Cathepsin K[18] confirmed our design hypothesis in that the 4-pyridine−carbonyl−leucine portion of **11** overlays with the Cbz-Leu portion of **4** in the unprimed region of the active site, and the terminal phenyl of the 4-phenoxyphenyl sulfonamide forms an aromatic−aromatic interaction with Trp 184 (Figure 2).

In conclusion, 1,3-bis(acylamino)-2-propanones are potent, reversible, and selective inhibitors of Cathepsin K. Opportunities to explore potential substrate-like binding interactions on both the primed and unprimed sides of the active site of other novel cysteine proteases readily presents itself through the use of 1,3-bis(acylamino)-2-propanone scaffolds and the well-developed synthetic methods of peptide chemistry. Moreover, further exploration of peptidomimetic approaches will follow from these studies in directing our efforts toward the discovery of drugs for the treatment of disease states such as osteoporosis.

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Supporting Information Available: Experimental details and structural characterization (4 pages). The coordinates have been deposited in the Brookhaven Protein Data Bank, accession numbers 1AU0 and 1AU2. See any current masthead page for ordering and Internet access instructions.

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(18) X-ray crystallography, mass spectrometry, and synthesis conditions are described in the Supporting Information.

(19) However, the observed orientation of the phenyl ring of the Cbz in **4** and Tyr 67 in the cocrystal with Cathepsin K was unanticipated; the aromatic−aromatic interaction occurs between the opposite edge of the phenol ring of Tyr 67 vs what was predicted on the basis of the binding conformation of leupeptin (**1**) in the papain cocrystal structure. Differences in crystal packing of the lattice may account for the difference in binding.