Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 1529–1534
Bioorganic & Medicinal Chemistry Letters

3,4-Disubstituted azetidinones as selective inhibitors of the cysteine protease cathepsin K. Exploring P3 elements for potency and selectivity

Eduardo L. Setti,* Dana Davis, James W. Janc, Douglas A. Jeffery, Harry Cheung and Walter Yu

Celera Genomics, 180 Kimball Way, South San Francisco, CA 94080, USA

Received 24 September 2004; revised 7 December 2004; accepted 20 December 2004
Available online 25 January 2005

Abstract—The synthesis of a series of highly potent and selective inhibitors of cathepsin K based on the 3,4-disubstituted azetidin-2-one warhead is reported. A high degree of potency and selectivity was achieved by introducing a basic nitrogen into the distal part of the P3 element of the molecule. Data from kinetic and mass spectrometry experiments are consistent with the interpretation that compounds of this series transiently acylate the sulfhydrile of cathepsin K.

Bone is a living tissue whose healthy structure depends on the maintenance of an adequate balance between the formation of the bone matrix by osteoblasts and the degradation of bone matrix by osteoclasts. Osteoporosis is a disease that results from an imbalance in activity between osteoblasts and osteoclasts and is characterized by a progressive decrease in bone density. There is mounting evidence that, at their site of attachment, osteoclasts release cathepsin K (cat K) and generate an acidic environment that facilitates demineralization and matrix degradation.[1] The fact that cat K has been shown to be highly and selectively expressed in osteoclasts[2] suggests that this specific protease may play a crucial role in bone resorption and may be a relevant target for therapeutic intervention to treat osteoporosis and related diseases.

Several chemotypes have been described as active-site directed inhibitors of cat K. Whereas epoxides,[3] vinyl sulfones,[4] chloromethyl,[5] and acyloxymethyl ketones[6] are reported to be irreversible inhibitors, nitriles,[7] aldehydes,[8] and ketones[9] are known to bind via a reversible covalent bond to the catalytic Cys-25. Recently, Zhou et.al.[10] have reported the synthesis of a series of 3-acylamino-azetidin-2-one derivatives as non selective cathepsin inhibitors. In a previous publication, we have shown that structures containing cyclic moieties as P2 elements and a 3,4-disubstituted azetidin-2-ones warheads, give rise to molecules that behave as selective and potent inhibitors of cat K.[11] In our continuing studies on the development of novel therapeutic agents to treat osteoporosis, we have synthesized a new series of 3,4-disubstituted azetidin-2-ones with the aim of identifying inhibitors with increased potency and selectivity, a good PK profile and suitability for in vivo evaluation.

Having identified the Ac6[12] moiety as the best P2 residue for selectivity, we decided to incorporate this structural motif into our inhibitors and, in an effort to further increase the binding affinity for cat K, to exploit known structural residues present in the S3 pocket of the enzyme. The possibility of designing P3 elements tailored to interact with Asp-61 has been one of our most promising approaches to obtain improved potency. This residue, located in the S3 subsite of the enzyme, is known to form ionic interactions with positively charged P3 moieties.[13]

In this article, we report the synthesis and inhibitory activity of a new series of highly potent and selective cat K inhibitors based on 3,4-disubstituted azetidin-2-ones. We also present data to assess the potential of this warhead in the development of new therapeutic agents to treat osteoporosis.

* Corresponding author. Fax: +1 650 866 6654; e-mail: eduardo.setti@celera.com

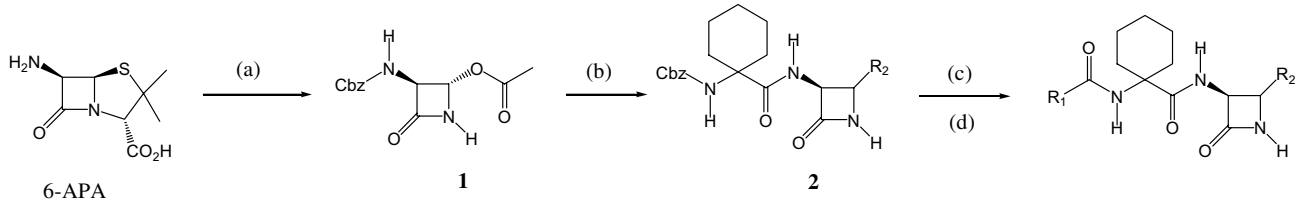
0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.12.088

The overall general strategy used for the synthesis of the compounds presented in Table 1 was based on the removal of the Cbz protecting group[14] of intermediate **2** and coupling the free amine to the corresponding acids, using HATU as an activating agent and diisopropylethylamine (DIPEA) as a base[15] (see Scheme 1). The intermediate **1** was obtained from the 6-aminopenicillanic acid (6-APA) according to procedures reported elsewhere.[16]

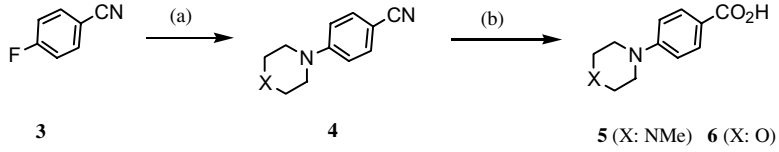
While the intermediate acids used in the synthesis of the biaryl analogues **24** and **25** were obtained from 4-fluorobenzonitrile (**3**) in two steps according to the sequence described in Scheme 2, the corresponding acid used in the synthesis of **27** was made following the synthetic sequence shown in Scheme 3. The intermediate acids required for the synthesis of the triaryl analogues **26**, **13**, **32**, **33**, and **34** were obtained according to reaction conditions depicted in Schemes 4–6, respectively.

Cat K inhibition and selectivity data are presented in Table 1.[17] As can be seen in these Tables, there was a significant improvement in selectivity profile, specially against cat S and cat L, when a basic nitrogen was introduced into the distal part of the P3 region of the molecule (see compounds **23**, **25**, **27**, **28**, **29**, and **32**). Potency against cat K was also increased specially when the basic nitrogen was incorporated into a large substituent, particularly evident in **29** and **32** since these inhibitors exhibited subnanomolar potency against cat K. The increased affinity of these two compounds toward cat K is believed to be the result of the presence of a positive charge combined with the particular shape of the P3 residue. This moiety presumably allows the positive charge of the nitrogen to perform an effective ionic binding interaction with the Asp-61 residue of the S3 pocket. Our assumption is based on the fact that crystallographic data obtained from compounds having the same P3–P2 moieties as compounds **23**, **28**, and **27**, attached to a nitrile based warhead,[7a] have shown that the distal nitrogen at the P3 elements is indeed interacting with the Asp-61 of the S3 region of the enzyme.[18] Unfortunately, attempts to crystallize the complex of cat K with any of the most soluble compounds of the azetidinone-based series have failed.

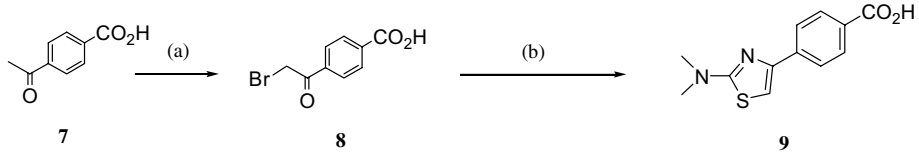
β-Lactams are well known inhibitors of serine proteases. Enzymes such as tryptase and elastase have been targeted for inhibition with compounds based upon this warhead.[19] This class of compounds exerts its mecha-



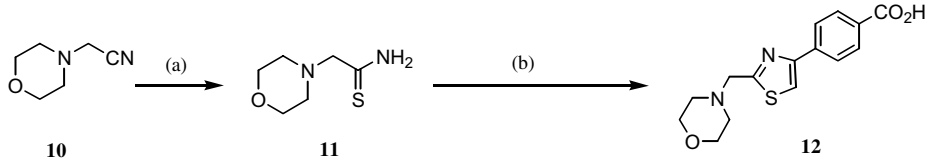
Scheme 1. Reagents: (a) Ref. 16a,b; (b) Ref. 11; (c) Pd/C*/H_2, EtOAc; (d) R_1CO_2H, HATU, DIPEA, DMF.



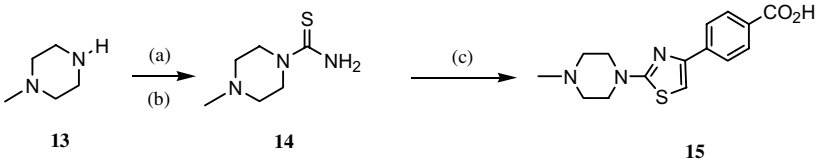
Scheme 2. Reagents and conditions: (a) *N*-methylpiperazine or morpholine, DMF, 100 °C; (b) HCl, 100 °C.



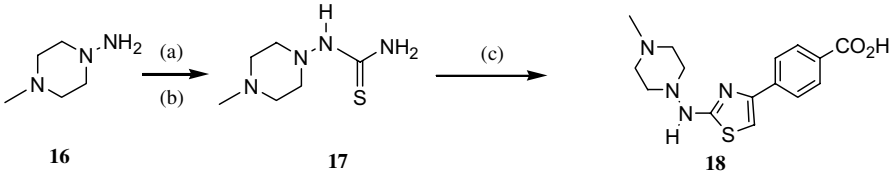
Scheme 3. Reagents and conditions: (a) AcOH, Br_2, 60 °C; (b) Me_2NCSNH_2, EtOH, 70 °C.



Scheme 4. Reagents and conditions: (a) pyridine, H_2S; (b) **8**, EtOH, 70 °C.

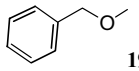
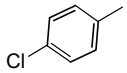
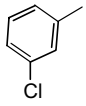
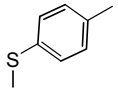
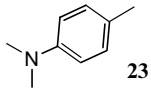
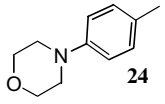
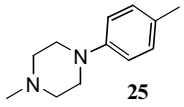
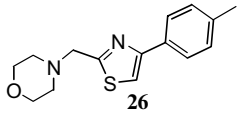
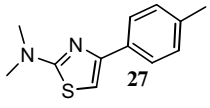


Scheme 5. Reagents and conditions: (a) thiophosgene, TEA, THF; (b) NH_4OH/MeOH; (c) **8**, EtOH, 70 °C.



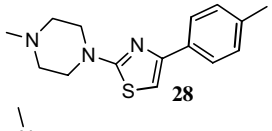
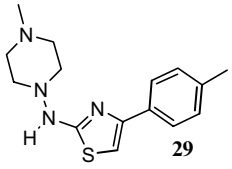
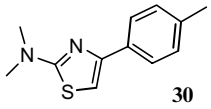
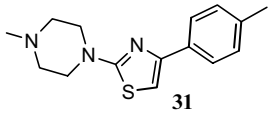
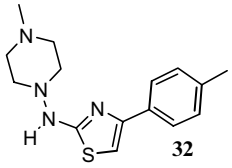
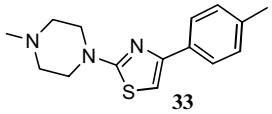
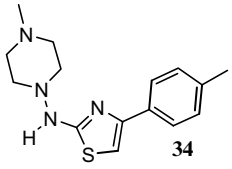
Scheme 6. Reagents and conditions: (a) thiophosgene, TEA, THF; (b) NH_4OH/MeOH; (c) **8**, EtOH, 70 °C.

Table 1. Cat K, B, S, and L inhibitory activity and selectivity

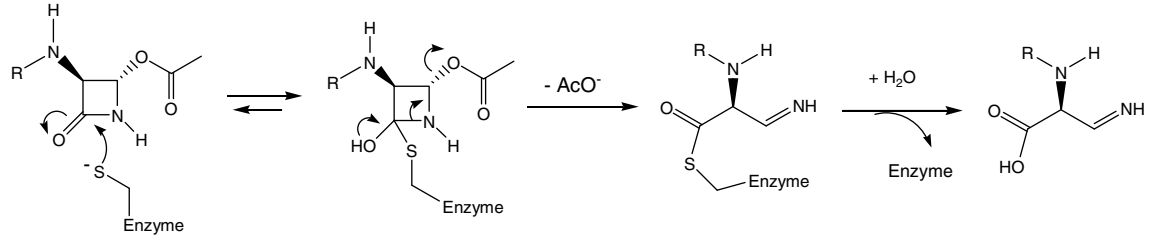
R_1	R_2	Cat K, K_i' (um)	Cat B, K_i' (um) (B/K)	Cat S, K_i' (uM) (S/K)	Cat L, K_i' (uM) (L/K)
 **19**		0.057	4.3 (75)	0.95 (16)	6.8 (119)
 **20**		0.092	2.1 (23)	3.4 (36)	8.2 (89)
 **21**		0.063	1.2 (19)	0.52 (8.2)	2.6 (41)
 **22**		0.012	1.2 (100)	3.5 (291)	6.4 (533)
 **23**		0.0031	0.53 (171)	2.8 (903)	2.1 (677)
 **24**		0.005	0.98 (196)	14 (2800)	19 (3800)
 **25**		0.0011	0.49 (445)	8.8 (8000)	1.4 (1273)
 **26**		0.0075	1.7 (227)	12 (1600)	11 (1467)
 **27**		0.0026	1.4 (538)	7.7 (2962)	0.39 (150)

(*continued on next page*)

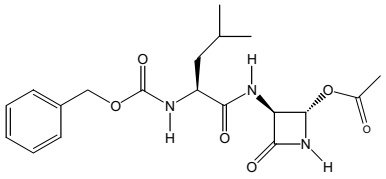
Table 1 (*continued*)

R_1	R_2	Cat K, K_i' (um)	Cat B, K_i' (um) (B/K)	Cat S, K_i' (uM) (S/K)	Cat L, K_i' (uM) (L/K)
 **28**		0.0011	0.94 (85)	8.5 (1636)	3.3 (3000)
 **29**		0.00025	0.091 (364)	8.8 (35,200)	2.1 (8400)
 **30**		0.018	25 (1389)	10.0 (556)	0.089 (5)
 **31**		0.0036	6.0 (1667)	51.0 (14,167)	1.1 (306)
 **32**		0.00026	6.0 (230,774)	140 (538,461)	0.31 (1192)
 **33**		0.0048	0.34 (71)	17.0 (3542)	2.4 (500)
 **34**		0.0082	5.2 (634)	150 (18,292)	8.2 (1000)

nism of inhibition through direct interaction of Ser-195 with the C-2 carbonyl of the β-lactam. The covalent participation of cathepsin K through Cys-25 should result in transient acylation of the enzyme as in Scheme 7. We examined the kinetics of inhibition of cathepsin K by CRA-13427 (Fig. 1) and representative compounds from Table 1. We typically observed slow on-set of inhibition consistent with a covalent mode of inhibition rather than simple rapid-equilibrium competitive inhibition. When cathepsin K was preincubated with CRA-13427, a fully inhibited species could be generated.[17] Following gel filtration chromatography, the inhibited species was isolated and the recovery of activity was monitored. We observed complete, time dependent recovery of cathepsin K activity. The recovery of activity was a first order process and in the case of CRA-13427 the $t_{1/2}$ was 26 min. This presumably corresponds to the rate of hydrolysis of the Cys-25 thioacyl enzyme intermediate to yield free cathepsin K (see Scheme 7).



Scheme 7. Proposed mechanism of inhibition of cat K by 3,4-disubstituted azetidin-2-ones.



CRA-13427

Figure 1.

To further substantiate whether CRA-13427 and cathepsin K formed a covalent adduct, we used mass spectrometry to measure the molecular weight of the enzyme before and after reaction with CRA-13427.[20] Untreated cathepsin K consisted of two species of approximate equal proportion with molecular weights of 23,711 and 23,768 Da. After incubation with CRA-13427, there were two additional species with molecular weights of 24,043 and 24,100 Da. This shift in molecular weight of 332 Da is consistent with the covalent attachment of one molecule of CRA-13427 to cathepsin K with the subsequent loss of acetic acid as outlined in Scheme 7. Approximately two-thirds of the cathepsin K was modified by CRA-13427 under these conditions. These results confirm that CRA-13427 forms a covalent bond with cathepsin K likely through transient acylation of Cys-25 during hydrolysis of the β-lactam.

Having identified several potent and selective cat K inhibitors, we decided to modify their structures in order to obtain molecules with potentially improved PK properties. To achieve this goal, we pursued the replacement of the acetyl group in the 4 position of the azetidin-2-one with a phenoxy group. In average, this structural change produced compounds with reduced cat K potency (compare compounds **27** and **30**, and **28** and **31**), being this change more noticeable in β (*cis*)-4-substituted analogues (compare compounds **28** and **33**, and **29** and **34**). This finding is opposite to what was reported by other researchers where the β-phenoxy-4-substituted analogues were more potent than their α-isomers.[10] Preliminary rat plasma stability studies showed that 4β-isomers were far more stable than the corresponding 4α-isomer. Therefore, rat pharmacokinetic parameters were measured for **33**. This compound when given intravenously, possessed high clearance (92 mL/min/kg), moderate volume of distribution (1.6 L/kg) and a short MRT (17 min) with a β $t_{1/2}$ (50%) of 21 min and α $t_{1/2}$ (50%) of 2 min. Consequently, considering their substrate-like mode of inhibition along with their poor PK profiles, we believe that 3,4-azetidin-2-ones moieties are unlikely to be suitable electrophiles to develop new therapeutic agents to treat osteoporosis.

In summary, the synthesis and evaluation of a novel series of potent and selective cat K inhibitors based on 3,4-azetidin-2-ones as warheads have been described. Combination of an Ac6 moiety in P2 and the presence of a basic nitrogen in the distal portion of the P3 of the molecule are essential to achieve a high degree of selectivity and potency. Initial data indicates that this class of compounds binds reversibly to the catalytic Cys-25 of cat K.

Acknowledgements

The authors wish to thank Merck & Co. for financial support.

References and notes

1. (a) Erlebacher, A.; Filvaroff, E. H.; Gitelman, S. E.; Derynck, R. *Cell* **1995**, *80*, 371; (b) Gowen, M. *Exp. Opin. Invest. Drugs* **1997**, *6*, 1999.
2. (a) Shi, G.; Chapman, H. A.; Bhairi, S. M.; DeLeeuw, C.; Reddy, V. Y.; Weiss, S. W. *FEBS Lett.* **1995**, *357*, 129; (b) Drake, F. H.; Dodds, R. A.; James, I. A.; Connor, J. R.; Debouck, C.; Richardson, S.; Lee-Rykaczewski, L.; Coleman, L.; Riemann, D.; Barthlow, R.; Hastings, G.; Gowen, M. *J. Biol. Chem.* **1996**, *271*, 12511.
3. Barrett, A. J.; Hanada, K. *Biochem. J.* **1982**, *201*, 189.
4. Palmer, J. T.; Rasnick, D.; Klaus, J. L.; Brömme, D. *J. Med. Chem.* **1995**, *38*, 3193.
5. Shaw, E. In *The Enzymes*; Boyer, P. D., Ed.; 3rd ed.; Academic: New York, 1970; Vol. 1, p 91.
6. Dai, Y.; Hedstrom, L.; Abeles, R. H. *Biochemistry* **2000**, *39*, 6498.
7. (a) Robichaud, J.; Oballa, R.; Prasit, P.; Falgueyret, J.-P.; Percival, M. D.; Wesolowski, G.; Rodan, S. B.; Kimmel, D.; Johnson, C.; Bryant, C.; Venkatraman, S.; Setti, E.; Mendonca, R.; Palmer, J. T. *J. Med. Chem.* **2003**, *46*, 3709; (b) Falgueyret, J.-P.; Oballa, R. M.; Okamoto, O.; Wesolowski, G.; Aubin, Y.; Rydzewski, R. M.; Prasit, P.; Riendeau, D.; Rodan, S. B.; Percival, M. D. *J. Med. Chem.* **2001**, *44*, 94.
8. Hanzlik, R. P.; Jacober, S. P.; Zygmunt, J. *Biochim. Biophys. Acta* **1991**, *1073*, 33.
9. (a) Marquis, R.; Yamashita, D.; Ru, Y.; Lo Castro, S.; Oh, H.; Erhard, K.; Des Jarlais, R.; Head, M.; Smith, W.; Zhao, B.; Janson, C.; Abdel-Meguid, S.; Tomaszek, T.; Levy, M.; Veber, D. *J. Med. Chem.* **1998**, *41*, 3563; (b) Marquis, R.; Ru, Y.; Lo Castro, S.; Zeng, J.; Yamashita, D.; Oh, H.; Erhard, K.; Davis, L.; Tomaszek, T.; Tew, D.; Salyers, K.; Proksch, J.; Ward, K.; Smith, B.; Levy, M.; Cummings, M.; Haltiwanger, R.; Trescher, G.; Wang, B.; Hemling, M.; Quinn, C.; Cheng, H.; Lin, F.; Smith, W. W.; Janson, C.; Zhao, B.; McQueney, M.; D'Alessio, K.; Lee, C.; Marzulli, A.; Dodds, R.; Blake, S.; Hwang, S.; James, I.; Gress, C.; Bradley, B.; Lark, M.; Gowen, M.; Veber, D. *J. Med. Chem.* **2001**, *44*, 1380; (c) McGrath, M. E.; Sprengeler, P. A.; Hill, C. M.; Martichonok, V.; Cheung, H.; Somoza, J. R.; Palmer, J. T.; Janc, J. A. *Biochemistry* **2003**, *42*, 15018.
10. Zhou, N. E.; Guo, D.; Thomas, G.; Reddy, A. V. N.; Kaleta, J.; Purisima, E.; Menard, R.; Micetich, R. G.; Singh, R. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 139.
11. Setti, E. L.; Davis, D. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2051.
12. Ac6 stands for alicyclic 1,1-disubstituted cyclohexyl.
13. McGrath, M. E.; Klaus, J. L.; Barnes, M. G.; Brömme, D. *Nat. Struct. Biol.* **1996**, *4*, 105.
14. *Standard procedure for hydrogenolysis*: to a solution of **2** (1 mmol) in ethyl acetate (15 mL), 10% Pd/C* (200 mg) was added. The mixture was hydrogenated at 50 psi for 8 h. The catalyst was separated by filtration through a short plug of Celite and the solution of the amine was used for coupling.
15. *Standard procedure for coupling*: to a solution of the corresponding acid (1 mmol) in DMF (10 mL), HATU (380 mg, 1 mmol), a solution of the free amine in ethyl acetate (15 mL) and diisopropylethylamine (209 μL,

1.2 mmol) were added at rt. The reaction mixture was stirred for 18 h at rt. The mixture was washed with satd $NaHCO_3$, dried over Na_2SO_4, evaporated, and the crude was purified on silica gel column, using a mixture of ethyl acetate/methanol. Satisfactory ^{1}HNMR and mass spectral data were obtained for all compounds.

16. (a) Arnould, J. C.; Pasquet, M. J. *Eur. J. Med. Chem.* **1992**, *27*, 131; (b) Singh, R.; Zhou, N. E.; Guo, D.; Micetich, R. Int. Pat. WO 98/12176, 1998.
17. Determination of apparent inhibition constants (K_i') were performed as follows. All enzymes used in these studies were produced by Celera Genomics with the exception of human cathepsin B. Human cathepsin B isolated from liver was obtained from Athens Research and Technology (Athens, GA). The substrates used in these studies were purchased from the following vendors: Z-Phe-Arg-AMC, Boc-Leu-Lys-Arg-AMC, and Z-Val-Val-Arg-AMC were from Bachem (Torrance, CA, USA) and Z-Leu-Arg-AMC was from Calbiochem-Novabiochem (San Diego, CA, USA). Typical rabbit cathepsin K, and human cathepsin L inhibition studies were performed in 50 mM MES (pH 5.5), 2.5 mM EDTA, 2.5 mM DTT, and 10% DMSO. The substrate used to monitor cathepsin K and L activity was Cbz-Phe-Arg-AMC. In both cases the substrate concentration was fixed at the K_m (40 μM for cathepsin K and 10 μM for cathepsin L). Cathepsin B inhibition studies were performed in 50 mM MES (pH 6.0), 2.5 mM EDTA, 2.5 mM DTT, 0.001% Tween-20, and 10% DMSO. The substrate used to monitor cathepsin B activity was Boc-Leu-Lys-Arg-AMC (supplied at the K_m, 190 μM). Cathepsin S inhibition studies were performed in 50 mM MES (pH 6.5), 2.5 mM EDTA, 100 mM NaCl, 2.5 mM 2-mercaptoethanol, 0.001% BSA, and 10% DMSO. The substrate used to monitor cathepsin S activity was Cbz-Val-Val-Arg-AMC (supplied at the K_m, 60 μM). Typically, the evaluation of a given inhibitor's potency was determined with the cathepsin of interest supplied at 1 nM (active-site concentration determined by titration with E-64). Enzyme was incubated with inhibitor, present at varying concentrations, for 30 min at room temperature (21–24 °C) in 96-well microtiter plates to allow for equilibrium to be achieved for slow binding inhibitors. After preincubation, reactions were initiated with the addition of the fluorogenic substrate specified above. The hydrolysis of this substrate yields AMC, which was monitored fluorometrically (filter pair: excitation 355 nm, emission 460 nm) using an FMAX Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). The velocity of the cathepsin-catalyzed reaction was obtained from the linear portion of the progress curves using a response factor of 275 RFU/μM (determined experimentally under the standard assay conditions with freshly prepared AMC stock solutions). Apparent inhibition constants, K_i', were calculated from the velocity data generated at the various inhibitor concentrations using the software package, Batch K_i (Biokin Ltd, Pullman, WA) [Kuzmic, P. *Anal. Biochem.* **1996**, *237*, 260, and; Kuzmic, P.; Sideris, S.; Cregar, L. M.; Elrod, K. C.; Rice, K. D.; Janc, J. W. *Anal. Biochem.* **2000**, *281*, 62]. Batch K_i provides a parametric method for the determination of inhibitor potency using a transformation of the tight binding inhibition model described by Morrison [Morrison, J. F. *Biochem. Biophys. Acta* **1969**, *185*, 269]. The apparent inhibition constant is related to the true thermodynamic binding constant, K_i, by the following relationship: $K_i = K_i'/(1 + [\text{substrate}]/K_m)$. Since substrates are typically supplied in the assays at their K_m, $K_i = K_i'/2$. The reversibility of rabbit cathepsin K inhibition by CRA-13427 was carried out by pre-forming the enzyme·inhibitor complex during a 30 min incubation phase. CRA-13427 was supplied at 25 nM during the incubation phase. Following the incubation phase, the reaction was diluted 50-fold into buffer containing substrate such that the final concentration of CRA-13427 was 0.5 nM. Alternatively, the enzyme·inhibitor complex was subjected to size exclusion chromatography using a BioRad BioSpin column according to the manufactures specifications. The recovery of activity of rabbit cathepsin K was monitored as a function of time and the $t_{1/2}$ for the recovery of enzyme activity (presumably corresponding to deacylation of Cysteine-25) was obtained by standard kinetic methods.
18. Unpublished results.
19. (a) Sykes, N. O.; Macdonald, S. J. F.; Page, M. I. *J. Med. Chem.* **2002**, *45*, 2850; (b) Cainelli, G.; Galletti, P.; Garbisa, S.; Giacomini, D.; Sator, L.; Quintavalla, A. *Bioorg. Med. Chem.* **2003**, *11*, 5391; (c) Sutton, J. C.; Bolton, S. A.; Davis, M. E.; Hartl, K. S.; Jacobson, B.; Mathur, A.; Ogletree, M. L.; Sulsarchyk, W. A.; Zahler, R.; Seiler, S. M.; Bisacchi, G. S. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2233.
20. Recombinant rabbit cathepsin K (16 μg) was denatured with 2.5 M guanadinium HCl and purified by standard reversed-phase HPLC on a Phenomenex Jupiter C5 column in water/acetonitrile plus 0.1% TFA. There was a single peak of absorbance at 280 nm that was collected. The molecular weight was measured by direct injection at 300 nL/min into an ABI QStar mass spectrometer. Data acquisition was performed in the multiple channel averaging (MCA) mode for 15–30 min. Rabbit cathepsin K that had been inhibited with CRA-13427 was treated exactly the same way and reversed-phase purification was carried out within 10 min of quenching the incubation with the addition of guanadinium HCl. The standard deviation among the charge species for the molecular weight measurements was ±10 ppm. The difference in mass for the two species in untreated cathepsin K is likely due to differential proteolytic processing that occurs during purification.